Mechanisms of Abdominal Pain in Paediatric Inflammatory Bowel Disease

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ABSTRACT

Introduction. Inflammatory bowel disease (IBD) is a condition affecting more than 3 million people in Europe and the USA combined. Patients report pain as one of the most severe and debilitating symptoms leading to a lower quality of life. Current analgesics lack efficacy for the treatment of visceral pain or produce unacceptable side effect profiles. New targets are needed.

Aims and methods. The aim of this thesis was to examine the activation of primary visceral afferents in C57BL/6 mice in response to biopsy supernatants from paediatric patients with IBD (Crohn's disease and ulcerative colitis) and functional abdominal pain syndrome (FAPS). By comparing the expression of pro-inflammatory and pro-nociceptive mediators in these biopsy samples with patient pain scores and afferent nerve recording activation, we identified putative mediators likely to be responsible for causing pain. The ability of inflammatory mediators to drive visceral nociception was then examined by their exogenous application in recordings of mouse and human visceral nociceptor activity.

Results. Nerve activation increased significantly in response to biopsy supernatants from FAPS, CD, and UC patients, when compared to controls. Supernatant IL-8, TNFα, IL-6 and IL-1β, levels were increased in IBD samples compared with control patients. Analysis of mRNA expression also showed high levels of pro-inflammatory cytokines and raised MMP-1, MMP-3, MMP-9, MMP-12, and MMP-19 in IBD samples. The expression of MMP-12 in biopsy samples from Crohn's patients significantly correlated with afferent firing suggesting a causative role. This was confirmed by exogenously application of MMP-12 stimulated afferent firing and sensitised responses to mechanical stimulation and inflammatory mediators. UC samples showed TIMP-1 as an effective inhibitor of afferent firing.

Conclusion. Data from this study demonstrates that the bowel of patients with IBD and FAP releases pro-nociceptive mediators which stimulate visceral afferents. MMP's play an important role in the afferent activation mediated by IBD samples suggesting that exploiting the endogenous inhibitor TIMP-1 could be a key target for future therapeutic strategies.

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STATEMENT OF ORIGINALITY

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LIST OF ABBREVIATIONS

5-HT	5-HYDROXYTRYPTAMINE (SEROTONIN)
ACC	ANTERIOR CINGULATE CORTEX
ADP	ADENOSINE-5'-DIPHOSPHATE
ASIC	ASID SENSING ION CHANNEL
ATP	ADENOSINE-5'-TRIPHOSPHATE
BK	BRADYKININ
CBT	COGNITIVE BEHAVIOURAL THERAPY
CD	CROHN'S DISEASE
CGRP	CALCITONIN GENE-RELATED PEPTIDE
COX	CYCLO-OXYGENASE
CRD	COLORECTAL DISTENSION
CRP	C-REACTIVE PROTEIN
DMSO	DIMETHYL SULFOXIDE
(c)DNA	(complementary) DEOXYRIBONUCLEIC ACID
(g)DNA	(genomic) DEOXYRIBONUCLEIC ACID
(ds)DNA	(double stranded) DEOXYRIBONUCLEIC ACID
DRG	DORSAL ROOT GANGLION
DSS	DEXTRAN SULFATE SODIUM
EC	ENTEROCHROMAFFIN CELL
EEN	EXCLUSIVE ENTERAL NUTRITION
EET	EPOXYEICOSATRIENOIC ACID
ENS	ENTERIC NERVOUS SYSTEM
ERK	EXTRACELLULAR SIGNAL REGULATED KINASE
EtOH	ETHANOL
FAPS	FUNCTIONAL ABDOMINAL PAIN SYNDROME
FGID	FUNCTIONAL GASTROINTESTINAL DISORDER

FODMAP	FERMENTABLE OLIGOSACCHARIDES DISACCHARIDES MONOSACCHARIDES AND POLYOLS
GI	GASTROINTESTINAL
GPCR	G-PROTEIN COUPLED RECEPTOR
IBS-C	IRRITABLE BOWEL SYNDROME-CONSTIPATION DOMINANT
IBS-D	IRRITABLE BOWEL SYNDROME-DIARRHOEA DOMINANT
IBS-M	IRRITABLE BOWEL SYNDROME-MIXED
IFAN	INTESTINOFUGAL AFFERENT NEURON
IFN-ɣ	INTERFERON GAMMA
IGLE	INTRAGANGLIONIC LAMINAR ENDING
IL	INTERLEUKIN
IPAN	INTRINSIC PRIMARY AFFERENT NEURON
IS	INFLAMMATORY SOUP
LC	LOCUS COERULEUS
MAPK	MITOGEN-ACTIVATED PROTEIN KINASE
MIA	MECHANICALLY INSENSITIVE AFFERENT
MMP	MATRIX METALLOPROTEINASE
Na_v	VOLTAGE GATED SODIUM CHANNEL
NOD	NUCLEOTIDE OLIGOMERISATION DOMAIN
NSAID	NON-STEROIDAL ANTI-INFLAMMATORY DRUG
P_2X	IONOTROPIC P2X RECEPTOR
P_2Y	METABOTROPIC P2Y RECEPTOR
PAR	PROTEASE-ACTIVATED RECEPTOR
PAG	PERIAQUEDUCTAL GRAY
PBMC	PERIPHERAL BLOOD MONONUCLEAR CELL
PI-IBS	POST-INFECTIOUS IRRITABLE BOWEL SYNDROME
(q)PCR	QUANTITATIVE POLYMERASE CHAIN REACTION

(RT)-PCR	REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION
PGE_2	PROSTAGLANDIN E2
PKA	PROTEIN KINASE A
PKC	PROTEIN KINASE C
PLC	PHOSPHOLIPASE C
RAP	RECURRENT ABDOMINAL PAIN
RCT	RANDOMISED CLINICAL TRIAL
(m)RNA	(messenger) RIBONUCLEIC ACID
RVM	ROSTRAL VENTROMEDIAL MEDULLA
SERT	SEROTONIN TRANSPORTER
SP	SUBSTANCE P
SSRI	SELECTIVE SEROTONIN REUPTAKE INHIBITOR
TCA	TRICYCLIC ANTIDEPRESSANT
TGFβ	TRANSFORMING GROWTH FACTOR BETA
TLR	TOLL-LIKE RECEPTOR
TNBS	TRINITROBENZENESULFONIC ACID SOLUTION
TNFα	TUMOUR NECROSIS FACTOR ALPHA
TREK	TWIK-RELATED POTASSIUM CHANNEL
TRPV	TRANSIENT RECEPTOR POTENTIAL CHANNEL VANILLOID
TRPA	TRANSIENT RECEPTOR POTENTIAL CHANNEL ANKYRIN
UC	ULCERATIVE COLITIS
UTP	URIDINE-5'-TRIPHOSPHATE
VFh	VON FREY HAIR
VmPO	VENTROMEDIAL POSTERIOR NUCLEUS
VMR	VISCEROMOTOR REFLEX
VPL	VENTRAL POSTEROLATERAL NUCLEUS
VPM	VENTRAL POSTEROMEDIAL NUCLEUS

CHAPTER 1: INTRODUCTION

1.1. Abdominal pain

Abdominal pain is one of the most common symptoms of GI diseases, accounting for over 16m outpatient visits each year in the USA alone (Peery, 2012). The aetiology of abdominal pain can be extremely varied and disease-specific. For example, GI-rated cancers such as pancreatic or colon cancer result in abdominal pain in 90-100% of patients, and often treatment with opiates yield minimal benefits (Caraceni, 1999; Freelove, 2006; Kroenke, 2010; Docherty, 2011). Abdominal pain resulting from appendicitis is responsible for approximately 50'000 cases in the UK (most require surgical intervention) with a lifetime incidence of 9% reported (Sellars, 2017). Inflammatory bowel disease (IBD), another leading cause of abdominal pain, shows increasing prevalence worldwide and evidence from patient symptom reports found that adults suffering with Crohn's disease rated abdominal pain as their most common and most prioritised symptom to be addressed (Bojic, 2016; Ghosh & Mitchell, 2007). An early onset of IBD in children can severely effect quality of life and increase the risk of persistence into adulthood (Walker, 2010). Compounding this, evidence suggests that conventional analgesics have minimal benefit in nearly half of IBD patients (Schirbel, 2010). Therefore, GI-specific analgesics are needed for symptoms of abdominal pain and new targets are needed.

A 2009 Cochrane review, analysing data from 9 clinical studies reported that abdominal pain in children in western societies could be as prevalent as 1 in 4 (Huertas-Ceballos, 2009). A recent meta-analysis of worldwide studies also showed similar prevalence reporting between 13-17% of children suffer from chronic abdominal pain (Korterink, 2015). Chronic abdominal pain, often recurring for months or years can significantly impact school and home life, affect sleep and appetite, and lead to psychological stress impacting quality of life (Warschburger, 2014; Chiou, 2010). The UK IBD audit found that over 83% of IBD paediatric patients suffered from abdominal pain with 52% of these patients stating that it was severe pain (Arnott, 2011). The authors also observed identical numbers in adults suggesting that the abdominal pain will persist into adulthood.

A study by Schirbel and colleagues reported that 40% of adult IBD patients endured high levels of pain during disease flare-ups, and 48% reported persistent pain lasting hours, with a further 10% reporting pain lasting for days (Schirbel, 2010). The researchers also noted that pain intensity increased with disease activity, and patients in remission still reported abdominal pain although it had the lowest pain intensity scores. Similar

levels of pain intensity were observed between UC and CD.

1.2. Epidemiology

It is reported that between 10-20% of children have abdominal pain in both Western and Eastern societies (O'Donohoe, 1996; Huang, 2000; Boey, 2001; Chitkara, 2005). Although no study focuses specifically on the prevalence of FAPS, the global prevalence of paediatric IBS is approximately 20%, with adult studies showing around 12%, suggesting a trend towards children, although the causes for these differences remain unknown (Karabulut, 2013; Lovell, 2012).

A meta analysis by Chitkara and colleagues (2005) identified FAPS as more prevalent in females than males with a 4:1 ratio. Interestingly, patients aged between 5-8 years old displayed a ratio of 1:1, which suggests a higher onset of FAPS in females during adolescence (Bode, 2003). A familial link also exists with the likelihood of a child developing a FGID significantly increased if one or more parent also suffered with a GI disorder (Bode, 2003). The long-term effects of FGID's can be difficult to quantify but suffering with a FGID during childhood can lead to a reduced quality of life, a higher number of school absences resulting in a drop in academic grades, and children with FAPS are also more likely to suffer with non-abdominal chronic pain as adults, suggesting a socioeconomic burden in addition to the clinical costs (Saps, 2009; Walker, 1998; Di Lorenzo, 2001).

1.3. Gastrointestinal diseases

Inflammatory bowel disease (IBD) is a term for multiple diseases, most commonly ulcerative colitis (UC) and Crohn's disease (CD), which affect approximately 120'000 and 90'000 people in the UK, respectively, and a combined total of 2.2m in Europe and 1.4m in the USA (Loftus, 2004). The less common subtype of intermediate colitis is reported in around 10% of IBD patients, where it shares symptoms and features of both UC and CD. The highest prevalence of IBD is seen in western populations such as Northern Europe and America, and has been shown to be as high as 246/100'000/year for UC and 214/100'000/year in CD, with the incidence for UC at 20.3/100'000/year and 15.6/100'000/year for CD (Loftus, 2004). This polarisation towards western societies has been linked with diet and lifestyle such as smoking and stress (NHS, 2013;

Ahuja & Tandon, 2010). In addition, it is widely accepted that a mixture of genetic factors, microbiome, autoimmunity, and local mucosal immune responses all contribute to increased susceptibility to developing IBD (Anderson, 2011; Mayer, 1990; MacDonald, 1995).

IBD is commonly diagnosed during adolescence but may present at any age where patients present with symptoms including but not restricted to bloody diarrhoea, abdominal pain, weight loss, and tiredness. IBD is a chronic condition leading to progressive deterioration of the GI tract and surgical intervention can be used as a last resort. Ulcerative colitis presents as a mucosal-based inflammatory disorder with rectum involvement in nearly 95% of patients (Kornbluth, 2004). It affects polymorphonuclear leukocytes and mononuclear cells (such as neutrophils, Th_2 cells, and monocytes) ultimately leading to crypt abscesses and goblet cell depletion (Burczynski, 2006; Gersemann, 2009). This goblet cell depletion is characteristic of UC and leads to a reduction in mucins and the mucus layer, which in turn has an impact on the epithelial barrier integrity which may play a role in the pathogenesis of UC. In contrast to this, CD often presents in the terminal ileum and colon, although it can affect any part of the GI tract. Endoscopic examination of CD shows scarring of the tissue called strictures, areas of 'healthy' tissue termed skip lesions, and inflammation affecting the full thickness of the intestinal wall. Symptoms such as weight loss, arthritis, and osteopenia (loss of bone mass) are all more common in CD than UC patients.

In addition to inflammatory diseases, functional bowel disorders also cause severe abdominal pain. As functional gastrointestinal disorders present without any clear organic changes in the gut upon endoscopic examination, patient symptoms and bowel habits are used as a basis for diagnosis using the ROME IV criteria (Drossman, 2016; Palsson, 2016). According to the ROME IV criteria, functional abdominal pain (FAP) should present with the following, once per week for at least two months; abdominal pain, insufficient criteria for any other functional gastrointestinal disorder, and the absence of any inflammatory, anatomic, metabolic or neoplastic processes. For IBS paediatric patients, the following must present at least once per week for 2 or more months; abdominal pain with 2 or more symptoms 25% of the time, including improvement with defacation, onset associated with change in stool frequency or appearance, and also the absence of any inflammatory, anatomic, metabolic or neoplastic processes (Drossman, 2016). Adult IBS is further stratified into constipation-, diarrhoea-prevalent, or mixed, whereas paediatric IBS and FAP have no further

stratification.

1.4. Aetiology of inflammatory bowel disease

Although the aetiology of IBD is still not fully understood detailed genetic and immunological studies have facilitated increased insight into the risk factors that contribute to a patient's susceptibility for the development of IBD. These studies have identified over 160 genes linked to IBD the majority of whom are found in both major IBD subtypes, Crohn's disease (CD) and ulcerative colitis (UC), although some variants are only found in CD or UC populations. For example, Jostins and colleagues recently identified 30 new genetic associations specific for CD and 23 specific for UC (Jostins, 2012). Additionally, authors have found an cumulative risk associated with the presence of a number of high risk genes (Jostins, 2012; Wang, 2013). Despite these findings, only a relatively small proportion of people carrying genetic risk factors for the development of IBD go on to develop the disease, suggesting that other environmental factors are also important for the development of pathology in genetically susceptible individuals. Smoking is one such example of the gene-environment interactions that increase the risk of developing IBD and exacerbates symptoms in CD patients (Jostins, 2012). Although by contrast in UC, smoking has been shown to be protective (Finnie, 1996). This affect has been attributed to the effect of smoking on mucin production, which is decreased in UC patients and elevated by smoking. Social status, geography, and diet have also been shown to have an association with the development of IBD (Sewell & Velayos, 2013; Reif, 1997). For example dietary modulation of the gut microbiome leading to increased diversity, complexity, variety, and number, of micro-organisms in the gut, has been shown to be linked to a reduced chance of developing IBD (David, 2014; Lee, 2015). With between 15'000-36'000 individual microbial species (10^{14} micro-organisms) in the gut, the microbiome is an important and complex environment. The balance between the gut microbiota and the hosts immune system is in a constant equilibrium aided by inhibitory signals towards the immune response. TLR and NOD-like receptors sense bacteria and contribute to the release of inhibitory cytokines such as TGFβ and IL-10 (Akagi, 2000). Changes in the balance of this interaction due to an increase or shift in the composition of the bacteria within the bowel leading to a loss in immunosuppression or increased immunoreactivity against the microbiota may contribute to a greater risk of chronic inflammation and IBD.

Loss of the epithelial barrier integrity is also a contributing factor to the development of IBD, whereby gut micro-organisms infiltrate the intestinal mucosal layer leading to an inflammatory response which ultimately fails to resolve. The importance of the microbiota in development of chronic inflammation in IBD patients has been underlined by faecal transplant studies that have shown resolution of inflammation in CD patients receiving a faecal transplant from healthy volunteers and inflammation in healthy volunteers following fecal transplant from a CD patient (D'Haens, 1998). A prospective study on antibiotic use in early life observed a greater risk of developing IBD with more frequent antibiotic use, further supporting the findings regarding the importance of gut bacteria composition and concentrations in maintaining a healthy gut environment (Hviid, 2011).

Although IBD is thought of as involving the innate rather than the adaptive immune response, with CD polarised towards Th_1 and Th_{17}-specific pathways, and UC a more Th_2-predominant response, a new hypothesis suggests that acute and chronic inflammation co-exist in an almost continuous cycle of re-initiation. Apoptosis, the normal process of cell death and recycling of intracellular components occurs naturally throughout the gut, but during chronic inflammation tissue necrosis also occurs (Nathan & Ding, 2010). This leads to exposure of intracellular components that otherwise would not be exposed, such as chromosomal DNA, RNA, and uric acid, which are recognised as foreign material and trigger an inflammatory reaction. Together with inflammatory processes brought on by gut micro-organisms, for example, this combination of inflammatory reactions has been termed 'unresolving inflammation' and is hypothesised to be a major driver in chronic inflammation (Nathan & Ding, 2010).

1.5. Clinically defining study patients

Functional gastrointestinal disorders (FGID's) incorporate a number of different disorders such as IBS, functional dyspepsia, functional abdominal pain syndrome (FAPS), and recurrent abdominal pain (RAP). In the late 1950's the term 'RAP syndrome of childhood' was first coined by the British pediatrician Dr John Apley which was characterised by 3 or more episodes of abdominal pain over a period of at least 3 months (Apley & Naish, 1958). This definition became part of what is now known as the ROME criteria. In 1999 the ROME II criteria outlined new definitions of FGID's but because it was found that up to 90% of RAP patients

could be classified as abdominal pain-predominant FAPS, the FGID's now conveyed IBS, functional dyspepsia, and FAPS (Rasquin, 1999; Walker, 2004). ROME III was introduced in 2006 and the clinical definitions became stricter leading to better patient stratification. From now IBS patients must present at least once per week for two or more months with; abdominal pain with two or more symptoms 25% of the time, an onset associated with change in stool frequency or appearance, and with the absence of any organic changes that would be responsible for this pain (Drossman, 2006). Whereas a FAPS diagnosis required episodic or continuous abdominal pain at least once per week for 2 months, no organic changes to the gut, and no criteria which would meet other FGID's in both adults and children (Drossman, 2006; Rasquin, 2006). Functional bowel habit changes are also assessed and some success has been had with anti-spasmodic medication.

Recently, the ROME IV criteria has been established. It has been expanded to more readily include multiple cultural differences, and to define further subgroups such as age-gender, and severity of symptoms including pain and discomfort (Drossman, 2016; Palsson, 2016). Some concerns have been raised that the more rigorous criteria leads to an overlap between disorders which could lead to confusion and possible exclusion of patients from studies which they could otherwise benefit from (Bai, 2017). As abdominal pain is a pre-requisite for biopsy collection, patients in this study are identified as FAPS while it is also recognised that patients may also present with IBS-like symptoms.

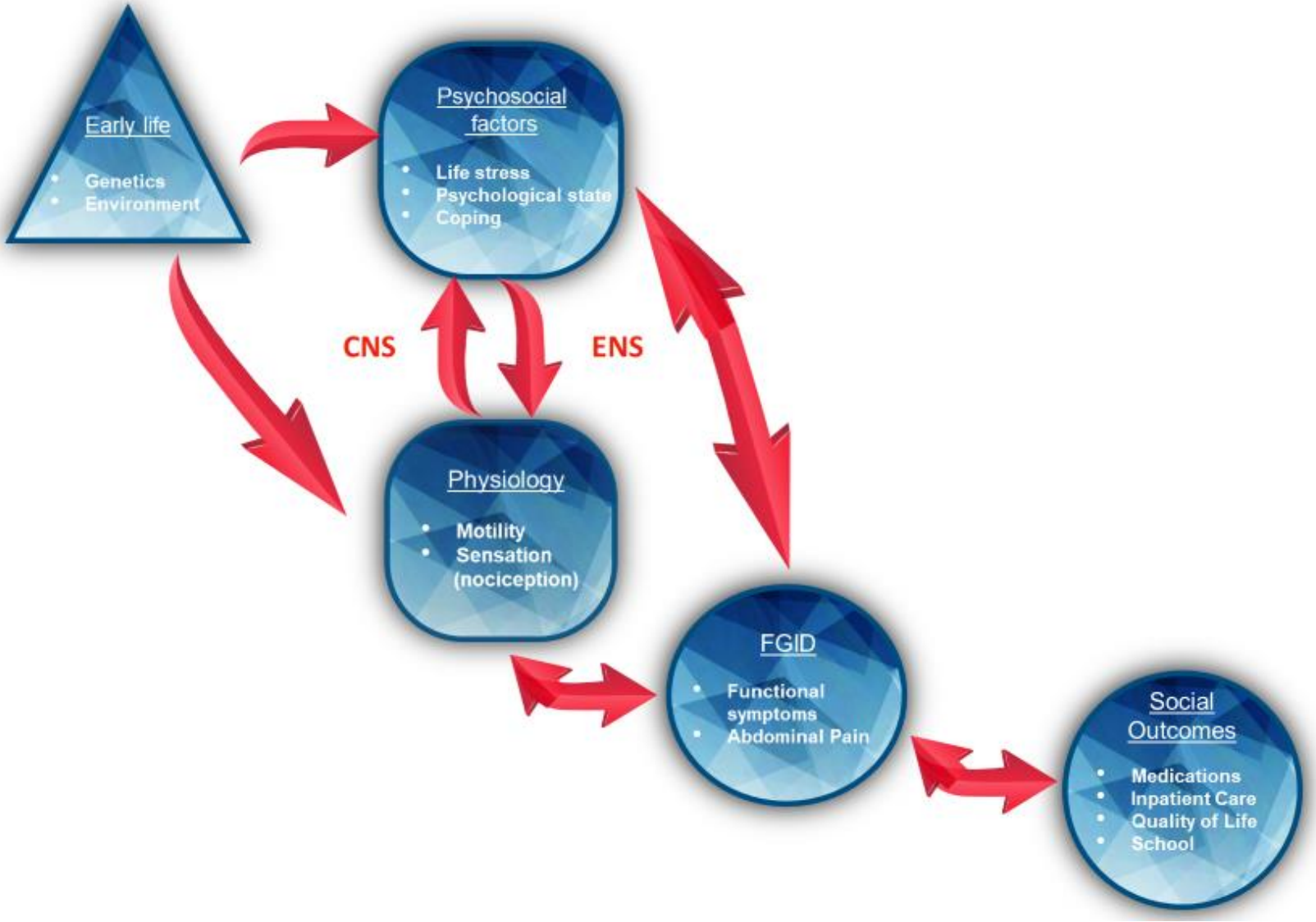
Table 1. Gastrointestinal diseases

Diagnosis	Gender ratio	Prevalence	Incidence	Clinical observations	Patient symptoms	Typical age of onset (years)	Regions affected
Crohn's disease (IBD)	1.4:1 (M:F)	214/100'000	15.6/100'000	strictures, skip lesions, full thickness inflammation, abscesses, malabsorption/ malnutrition, perforated bowel	abdominal pain, weight loss, bloody diarrhoea, fatigue, arthrits, osteopenia	15-35 (early onset 0-5)	Terminal ileum, ileocaecal regions, small intestine (ileitis), large intestine, oesophagus/stomach/duodenum (gastroduodenal), mouth
Ulcerative colitis (IBD)	1:1 (M:F)	246/100'000	20.3/100'000	mucosal inflammation, goblet cell depletion, crypt abscesses, toxic megacolon	abdominal pain, weight loss, bloody diarrhoea, fatigue, fever, loss of appetite, rectal bleeding,	15-35 (early onset 0-7)	Large intestine, rectum
Indeterminate colitis (IBD)	1:1.1 (M:F)	20/100'000	2.3/100'000	goblet cell depletion, crypt abscesses, focal/ diffuse mucosal inflammation, V shaped clefts	abdominal pain, persistent diarrhoea, rectal bleeding, fever, fatigue	35-55	Large intestine
Microscopic IBD	1:5.6 (M:F)	103/100'000	21.2/100'000	inflammation visible under microscope, commonly associated with coeliac disease and diabetes	diarrhoea (no blood), abdominal pain, weight loss, incontinence, fatigue, bloating, joint/ muscle pain	50-60	Large intestine, rectum
IBS-C	1:2 (M:F)	3920/100'000	1000-2000/100'000	abdominal pain (3 days/ month for 3 months), loose stool ≧25% time, visceral hypersensitivity	abdominal pain, bloating, diarrhoea, nausea, incomplete evacuation, increased urge	15-45	Large intestine
IBS-D	1.4:1 (M:F)	5560/100'000	1000-2000/100'000	abdominal pain (3 days/ month for 3 months), hard stool ≧25% time,	abdominal pain, bloating, constipation, nausea	15-45	Large intestine
IBS-M	1:1 (M:F)	2580/100'000	unknown	abdominal pain (3 days/ month for 3 months), hard stool ≧25% time, loose stool ≧25% time	abdominal pain, bloating, constipation, nausea, diarrhoea	15-45	Large intestine
PI-IBS	1:1 (M:F)	10'000-30'000/100'000 post gastroenteritis	10'000/100'000 post gastroenteritis	previous gastroenteritis, visceral hypersensitivity, abdominal pain (3 days/ month for 3 months), bloating	abdominal pain, bloating, constipation, nausea,	unknown	Large intestine
FAP	1:1.5 (M:F)	2000/100/000	unknown	abdominal pain once per week for 2 months, absence of toher GI disorders	abdominal pain	<16	Large intestine

1.6. The biopsychosocial model of pain

Although the causes of FGID's are unknown, relationships between psychosocial factors and the prevalence of disease has been studied. The signals relayed between the brain and the gut, termed the brain-gut axis, feature heavily in chronic abdominal pain. FGID's have been associated with a patient history of abuse or early life trauma, and a correlation between the duration of abuse and an increased risk of developing a GI disorder has been shown (Walker, 2010). Figure 1 shows the biopsychosocial model of pain in FGID's which incorporates a range of psychosocial factors that can affect physiological function such as a concurrent psychiatric disorder, catastrophising, and increased autonomic functioning, for example, GI motility (Talley, 1998; Drossman, 2000, Tanaka, 2008). Further analysis of these psychological factors and their relation to abdominal pain have revealed an increased prevalence (40-60%) of psychiatric disorders in patients with FGID's, with the most common being anxiety and depression (Pinto-Sanchez, 2015; von Gontard, 2015). A small study involving children with recurrent abdominal pain (RAP) found that nearly 80% suffered with concurrent anxiety and 45% suffered with depression and it has been suggested that children with RAP are more likely to experience greater levels of pain from normal daily stresses (Campo, 2004; Walker, 2010). These two observations likely converge into themselves in a feedback loop that encourages the other, where greater levels of anxiety result in greater levels of abdominal pain which then lead to greater levels of stress. The observation that stress induces changes in the gut motility, secretion, visceral sensitivity, and local inflammatory responses, emphasises the role of the brain-gut axis (Huerto-Franco, 2012; Moloney, 2015; Stanisor, 2013).

Figure 1. The biopsychosocial model of pain



1.7. Current treatment options for abdominal pain

The goal of this study was to focus on the peripheral activation of nociceptors for GI disease with chronic abdominal pain. Although there are several peripheral and central regions in which therapies can act, this study centered around mechanisms involved in generating nociception at the peripheral afferent with any future peripheral treatment option benefiting from this study. However, several treatment options currently exist and are discussed below.

1.7.1. Pharmacological intervention

Pharmacological interventions aimed at the central nervous system have seen moderate benefits in clinical trials. A Cochrane review by Rupert *et al*. in 2011 looked at 6 clinical trials assessing the benefits of either tricyclic antidepressants (TCA) or selective serotonin re-uptake inhibitors (SSRI's) on abdominal pain in adult IBS patients. They reported that there was a statistically significant improvement in pain levels when patients were on TCA's but noted less of an effect from SSRI's, with other studies also noting the analgesic effects to be mild (Rupert, 2011; Tack, 2006; Sindrup, 2005).

Pharmacological treatments that act specifically in the periphery are both varied and complex. Linaclotide, a guanylate cyclase C agonist has recently been approved for the treatment of constipation predominant IBS (IBS-C) and shows significant efficacy against pain endpoints (Johnston, 2013). Currently, treatments such as linaclotide have become available for IBS-C patients to combat severe symptoms such as abdominal pain. Linaclotide improves motility by increasing the secretion of chloride and bicarbonate ions while inhibiting sodium ion absorption by binding to the GC-C receptor on the surface of intestinal enterocytes causing changes in downstream messenger levels to regulate intestinal processes (Busby, 2010). The effects of this mechanism on visceral pain have been studied in colorectal afferents in rodents where noxious mechanical responses were significantly reduced in the presence of linaclotide (Feng, 2013, Castro, 2013). Phase III trials showed that approximately 50-60% of patients reported a beneficial effect on abdominal pain (Rao, 2012; Chey, 2012; Rao, 2014). However, such treatments have not yet been targeted towards paediatric patients, and adverse effects such as diarrhoea is common. Although beneficial to some patients, therapies for a broader range of patients including paediatrics are still needed.

In addition, a Cochrane report reviewing the beneficial effects of antispasmodics such as mebeverine on abdominal pain in IBS patients, suggested that there was a significant improvement in pain levels with treatment (Rupert, 2011). However, many clinical trials studying the effects of antispasmodics were undertaken with less than optimal patient numbers and meta-analysis of the data suggested that due to the diversity in antispasmodic drugs in clinical use, the number of reproducible trials for specific treatments was lacking and conclusions based on these reports are not fully conclusive (Rupert, 2011).

Synthetic glucocorticoids such as prednisolone offer an effective solution to reducing gut inflammation leading to a reduction in abdominal pain. Glucocorticoids are highly lipophilic meaning that they pass through cellular membranes to bind to glucocorticoid receptors within the cytoplasm causing dissociation of chaperone proteins to internalise receptors.

Biological agents such as infliximab, golimumab, and adalimumab are monoclonal antibodies that target both soluble and membrane-bound TNFα preventing TNF_{R1} binding and activation. Although infliximab is the first choice biologic for IBD, particularly CD, *in vitro* data has demonstrated much greater binding affinity for golimumab compared with infliximab and adalimumab, although this has not necessarily been shown in the clinical responses. Although infliximab is effective in many CD patients at resolving severe inflammation, the effects on abdominal pain are reliant on the suppression of the immune response which can take weeks or months to control. Infliximab is a chimeric monoclonal antibody with TNFα binding regions. It has a half-life of 9.5 days meaning that after initial frequent dosing, a maintenance regimen of 8 weeks between inpatient visits validate it as a cost-efficient therapy. Several recent meta-anlyses have been conducted to collate and assess the vast number of clinical studies using infliximab. It is currently the preferred biologic treatment in IBD, and studies which look to understand the efficacy and side effect profiles of all biologics currently in clinical use demonstrate a superior efficiency with infliximab compared with others and found that in UC patients with no history of exposure to biologic, there was a greater probability of inducing clinical remission and mucosal healing compared with other biologics such as adalimumab. A further study reviewing the recent data of infliximab also observed a superior effect in both CD and UC with a reduced rate of hospitalization and surgery (Mao, 2017).

Based on promising *in vitro* studies with golimumab, a series of multi-center RCT's have been published from

the program of ulcerative colitis research studies utilizing an investigational treatment (PURSUIT), where over 1000 patients have been used to assess golimumab in UC patients (Sandborn, 2014). Overall, the PURSUIT series of studies have demonstrated promising safety, tolerability, and treatment effects in moderate-severe UC patients. At present, golimumab is indicated for use in active UC as there are yet to be any published trial data for use in CD patients. Retrospective observational studies in CD have demonstrated a clinically relevant response to treatment when applying the same dosing regimen as UC patients (Martineau, 2017). Trials to compare the response in UC with CD could prove clinically significant in the future.

Pharmacotherapies are often complicated by mixed results and contraindications, whereas cognitive behavioural therapy (CBT) and hypnosis can be applied to almost all patients regardless of concurrent treatments due to limited adverse effects. Cognitive behavioural therapy has been shown to benefit patients suffering with chronic abdominal pain, although the high cost and intensive patient interaction means that its availability for large patient cohorts will be limited (Drossman, 2003; Lackner, 2008). The results however are promising. A clinical trial by Simrén and colleagues in 2004 demonstrated efficacy of hypnotherapy in a subset of IBS patients and a more recent study found that patient satisfaction was rated highly in nearly 70% of patients using hypnotherapy (Simrén, 2004; Lindorfs, 2013; Simmer, 2004).

1.7.2. Diet

Recently, emphasis has been placed on understanding the benefits of diet and supplementation in IBS patients. Observational studies have attempted to understand dietary influences that may contribute to an increased risk of IBD.

The most recent intervention is the FODMAP diet - fermentable oligosaccharide, disaccharide, monosaccharide, and polyol restricted diet. Short chain carbohydrates can be fermented in the colon due to limited absorption in the small intestine, impacting on already prominent symptoms such as bloating. Research in children with FGID's have assessed low FODMAP diets with encouraging results. By reducing FODMAPs patients reported a reduction in abdominal pain severity and a reduced amount of nausea and bloating (Chumpitazi, 2014). Further small-scale studies have also shown promising results although larger

trials will be needed to properly assess any benefits, in particular the improvement of pain (Staudacher, 2011; Halmos, 2014).

Probiotics, such as *lactobacillus, bacillus subtilis,* and *streptococcus faecium,* have been studied for their beneficial effects on IBS symptoms such as bloating and abdominal pain, and some trials report increased quality of life due to symptom relief, although there remains ongoing debate regarding the effect size and the precise cocktail of bacteria required for specific symptoms (Ford, 2014, Choi, 2015).

A greater risk of IBD has been linked with higher amounts of polyunsaturated fatty acids and omega-6 fatty acids (found in meats and fats), and a reduced risk when diets consist of fruits, fiber, and vegetables.

Vitamin D deficiency is common amongst IBD patients which has led to researchers testing whether targeted high-dose vitamin D treatment could be beneficial. An observational study by Pappa (2014) revealed that IBD patients who corrected this deficiency were significantly less likely to require surgery than patients who did not supplement vitamin D levels. An encouraging small RCT demonstrated a recued risk of relapse in CD patients with daily high-dose vitamin D (1200IU) compared with a placebo group, although a large scale trial would be needed to confirm these findings.

Exclusive enteral nutrition (EEN) is a formula diet commonly administered for 4-12 weeks and has been consistently shown to induce remission in CD patients where it is associated with mucosal healing and when tested with children suffering from CD EEN was as effective a corticosteroids at inducing clinical remission and more effective for mucosal healing over a 10-week period (MacLellan, 2017).

1.7.3. Surgical intervention

Around 70-90% of CD patients will need surgery throughout the course of the disease to limit symptoms and further tissue destruction (Hwang, 2008). Surgery often involves resection of the rectum and colon in an ileal pouch-anal anastomosis. Similar surgical techniques are used in UC patients although the total proctocolectomy with end ileostomy remains the gold standard, which limits any further bowel function symptoms and any additional risk of colon cancer (by removing the colon and rectum). Within both CD and UC, the restorative proctocolectomy with ileal pouch-anal anastomosis is commonly preferred, as it removes tissue but saves the defecation pathway, however, ongoing management and further surgery is a

consideration.

1.7.4. Future therapies and current clinical trials

Current clinical trials are providing promising results for future treatments of abdominal pain. The IRIS-2 phase II clinical trial assessing the effects of 10mg Ibodutant, a potent neurokinin-2 receptor antagonist showed improved abdominal pain in 47% of adult IBS-D patients over 8 weeks (Tack, 2015). A further phase III trial could show reassuring results. The H_1 antagonist ebastine was tested in a 12 week study where painful symptoms decreased in 46% of patients compared with only 12% in the placebo group, although the long-term benefits remain to be understood (Wouters, 2016).

A study currently underway is attempting to address the availability issues with CBT whereby 10 weekly online modules are delivered to children with FAP (Olen, 2017). The trial aims to understand if CBT is effective when delivered remotely and measures key endpoints such as quality of life, and pain scores. The results are due to be published in the coming years.

Although only targeting symptomatic relief, a new combination of herbal and natural ingredients such as curcumin and peppermint oil, has recently provided encouraging results in a small-scale 8-week trial involving adult IBS patient (Alt, 2017). Over 60% of patients within the treatment arm reported a 30% reduction in abdominal pain which was significantly greater than the placebo group. No adverse effects or safety concerns were raised and a larger scale trial could provide promising results.

A small-scale clinical trial assessing the influence of l*actobacillus reuteri* DSM 17938 on abdominal pain in FAPS and IBS paediatric patients recently found that there was a significant benefit on abdominal pain in both patient groups when in the treatment arm (Jadrešin, 2017). However, the small patient numbers (*n*=50) mean that there is a potential for underpowering the study and type I statistical errors, especially when comparing with placebo effects. Despite this, the results appear promising but further studies would be needed to assess l*actobacillus reuteri* in a broader population.

1.7.5. Cost

The cost of care of abdominal disorders is significant. Although this cost incorporates many functions of both in-patient and out-patient care, visceral pain can make a large contribution to overall treatment cost. Nearly half of children who suffer with FAP will continue to have abdominal pain as adults along with an increased likelihood of clinical depression (Creed, 2001; Horst, 2014). Therefore addressing abdominal pain in early life may reduce the probability of a multi-factorial chronic disease and reduce long-term costs. In the USA, more than $20b of medical care was attributed to adult FGID's such as IBS, largely due to expensive colonoscopy procedures (Dhroove, 2010). In UC, Bassi and colleagues reported that a patient in remission could cost around £1000 per year, and this could increase 4-fold to more than £4000 if the patient suffers more than 2 relapses in a year (Bassi, 2014). Recent studies found similar costs for FGID's which have a high symptomatic prevalence of severe abdominal pain, and of which generated annual costs per patient of around €2500 in Europe, and over $6000 in the USA (Hoekman, 2015; Dhroove, 2010). As a proportion of abdominal pain-related medical costs, FGID's account for a large percentage. For example, in the Netherlands, abdominal pain results in €620m of medical care, of which approximately €220m is for FAP (Jansen, 2014).

With high costs and multiple analgesic treatments often providing limited efficacy, hospitalisation due to abdominal pain and life-long after-care means that the need for new and more targeted visceral pain treatments is needed.

1.8. Patient drugs

1.8.1. Mebeverine

Introduced in 1965, Mebeverine is now in common use for the treatment of functional symptoms in IBS such as diarrhoea and bloating. It has potent properties through actions on muscarinic receptors to induce smooth muscle relaxation. Anti-spasmodics such as Mebeverine have resulted in better regulation of bowel motility in patients although the effects on abdominal pain are minimal. A 2014 trial in children with FAPS demonstrated good tolerability of anti-spasmodics, but only marginal benefits in pain relief in the treatment group after 12 weeks (Pourmoghaddas, 2014). This was also apparent in a meta analysis involving over 2500 patients which indicated only a small benefit to pain relief with Mebeverine and several other anti-

spasmodics (Martínez-Vázquez, 2012). Mebeverine, along with Alverine and Pinaverium are favoured because of their limited side-effect profile and proven safety and tolerability record for motility symptoms. Further trials are underway currently to understand the benefit of Mebeverine on paediatric FAPS patients but no results have been released as yet (EU Trials Register: 2015-0032293-32)

1.8.2. Paracetamol

Paracetamol is widely used amongst patients with IBS for its analgesic effects although it has limited efficacy in IBD patients. It is used as a first-line analgesic for a range of pain conditions such as oral pain, arthritic pain, muscular pain, and tension headaches (Graham, 2013). Paracetamol acts within the peripheral and central nervous system by reducing PG synthesis through inhibition of cyclo-oxygenase (COX) enzymes, specifically COX-2. Although there is little published data on the efficacy of paracetamol in paediatric FGID patients, adult IBS data suggests analgesic benefits (Locke, 2000). A 2015 meta analysis which examined a range of acute and chronic pain conditions found that while paracetamol reduced overall pain, the efficacy is dependent on the pain phenotype, with more chronic pain conditions yielding minimal benefits (Moore, 2015). Paracetamol remains one of the most used analgesics worldwide due to a high tolerance and low toxicity along with limited contraindications with other medications, however the benefits of paracetamol as a visceral pain-specific therapy are limited.

1.8.3. Ibuprofen

Ibuprofen is a class of NSAID's that is commonly prescribed for inflammation and a number of acute and chronic pain conditions such as dental pain, headache and migraine, osteoarthritis, and post-surgical treatment (Perrott, 2004; Sarzi-Puttini, 2013; Bailey, 2014). Its anti-inflammatory properties act primarily through reducing the activity of COX-1 and COX-2; the downstream enzymes from the arachidonic acid pathway resulting in limited PG synthesis, but this mechanism is also responsible for reported adverse effects such as alteration of platelet function and GI mucosal damage and bleeding (Lichtenberger, 2012; Fukushima, 2014). The GI interference is concerning when given to patients already suffering with GI related diseases. The resulting GI mucosal damage has been shown to be a result of increased neutrophils attracted to vascular

endothelial cells resulting in the release of free radicals and proteases causing further damage (Wallace, 1990). Although prescribing ibuprofen to paediatric FGID patients has few contraindications, when combined with warfarin, beta-blockers, ACE inhibitors, and anti-hypertensives, further complications can arise (Pierce, 2010; Moss, 2014). In general however, there is a good safety and tolerance record with ibuprofen, although the analgesic properties are limited and are best suited for mild pain limiting its use for more severe visceral pain.

Table 2. Patient drugs

Drug Class	Example Drug	Mechanism of Action	Clinical Use	Limitations
Anti-spasmodic	Mebevrine	Antimuscarinic. Targets muscarinic receptors to induce smooth muscle relaxation	IBS. Reduce abdominal cramps, and gut motility problems	Obstruction/ worsening of gut dysmobility, such as constipation
Cox-inhibitor	Paracetamol	Mechanisms largely unknown but acts centrally to target COX-2 enzymes and prostaglandin synthesis	IBS. General mild-moderate analgesic and anti-pyretic	Limited analgesia in moderate to severe visceral pain
NSAID	Ibuprofen	Non-selective COX inhibitor	IBS. Analgesic and anti-inflammatory in GI tract and used for musculoskeletal pain	Gastric bleeding
Biologic Agents	Infliximab	Anti-TNF-alpha antibody targeting soluble and membrane-bound TNF-alpha to stop the binding to receptor	IBD. Effective in many CD patients but also has been used successfully in UC patients to limit disease	Aggressive strategy, opportunistic infections, time of onset of therapy still debated
Synthetic glucocorticoids	Prednisolone	Highly lipophilic so passes through membranes to bind to glucocorticoid receptors in cytoplasm causing internalisation of receptors.	IBD. Reduce inflammation	Approximately one third of patients will not respond to this treatment
Immunosuppressants	Azathioprine	Pro-drug, converted to 6-mercaptopurine to inhibit proliferation and activation of lymphocytes	CD. Long-term use	Only effective in approximately 40-50% of patients
5-ASA	Mesalzine	Mechanism largely unknown but contributes to reducing free radical damage in the gut	UC. Reduce inflammation	Worsening of GI problems such as diarrhoea and abdominal pain, nausea also reported

Table 3. Available treatments for pain GI diseases

Drug Class	Example Drug	Mechanism of Action	Clinical Use	Limitations
Centrally-acting pharmacological	Opiates (morphine, tramadol, hydromorphone)	Modulates opioid receptors in the CNS	Severe pain and post-operative pain	Nausea, vomiting, constipation, addiction
	Calcium channel modulators (gabapentin, pregabalin)	Valium channel modulation on nociception	Neuropathic pain	No extensive evidence of efficacy in IBD or IBS
Peripherally-acting pharmacological	Guanylate cyclase C agonist (Linaclotide)	Guanylate cyclase C agonist to increase amount of fluid in intestines	IBS. constipation	Adult IBS only
	5-HT receptor antagonist (Alosetron)	5-HT3 receptor inhibitory action	IBS-D - Effective in reducing abominal pain	Specific to IBS-D subtype
Anti-microbial	Antibiotics (rifaximin, ciproflaxin)	Inhibit bacterialRNA synthesis	IBD	Lacking in UC trials, optimising combinations is often necessary
Psychotropic	TCA's (amitryptyline, desipramine)	Non-selective SNRI's and 5-HT inhibitor	IBS. May decrease comorbidities of anxiety and depression	Inconclusive regarding patient suitability and efficacy
	SSRI's	Inhibitis SERT	IBS. May decrease comorbidities of anxiety and depression	Long delay in treatment effect
	Cognitive behaviour therapy	Psychological	Abdominal pain	High cost and intensive patient interaction
	Hypnotherapy	Psychological	Abdominal pain	High cost and intensive patient interaction
Diet	Probiotics (lactobacillus, bifidobacterium)	Improve microbial balance in gut	UC. IBS. Abdominal pain and bloating	Optimum bacteria and dosing not well understood
	FODMAP	Influence on gut microbia	IBS	Accurate clinical investigations lacking, large scale trials needed
Surgical	Resection, total or restrictive proctocolectomy	Removal of affected areas in gut	IBD	Inherent risks with surgery, post-operative complications such as parastomal herniation, pouchitis
Biologics	Infliximab, Golimumab, Adalimumab, Vedolizumab	TNF inhibition	CD and UC	Hospitalisation required
Leukocyte treatment	Adacolumn	Blood is filtered through an adacolumn removing granulocytes, monocytes and macrophages	UC	Hospital visit required, invasive

1.9. Neuroanatomy of the GI tract

1.9.1. The enteric nervous system

The intrinsic efferent and afferent neurons of the enteric nervous system (ENS) control many processes such as mucosal secretion and absorption, and local blood flow, and they permit bidirectional communication between the brain and the gut. In general, nearly half of the innervation from the ENS is located in the myenteric neurons (30%) and submucosal neurons (14%) which project to the villi and branch within the submucosal and myenteric ganglia (Sanders & Smith, 1986; Wilson, 1987; Furness, 2000). Multi-axonal, polymodal afferents called intrinsic primary afferent neurons (IPANs) innervate various layers of the intestine. They respond to short chain fatty acids, hormones, glucose, and distension of the intestinal wall (Furness, 1998). The mucosal layer IPANs lead to the lamina propria, and submucosal IPANs connect extensively with other submucosal neurons and myenteric ganglia, which have further connections with interneurons, motor neurons, and other IPANs. The enteric interneurons link with other ascending interneurons to form chains capable of communicating the inputs from IPANs. They are an important class of intrinsic peptidergic neurons as they also contain endogenous opioids important in pain relief (Brookes, 1997). Secretomotor and vasomotor neurons represent only a small proportion, around 2%, which project to the mucosa from the myenteric ganglia (to induce secretions or control the diameter of blood vessels, respectively).

The excitatory and inhibitory circular muscle motor neurons are also an important class of neuronal projections within the ENS. Ending deep within the muscular plexus, these neuronal projections are stratified into inhibitory and excitatory. Longitudinal muscle motor neurons, representing around 25% of neuronal projections, are mostly small diameter neurons which receive synaptic inputs from the IPANs and the descending efferent pathways (Brookes, 1992).

Another type of neuron exists termed the intestinofugal afferent neuron (IFAN) which has its cell body located in the myenteric plexus and projects to the sympathetic prevertebral ganglion where

it synapses with post-ganglionic neurons. IFAN's are mechanoreceptors within the gut, responding to physiological stretch, with many projections synapsing with IPANs (Furness, 2000). The IFAN's are responsible for reducing gut motility and secretion in response to gut distension and therefore facilitate physiological stretching to prevent excessive intraluminal pressure after meals.

The extrinsic innervation of the GI tract is broadly divided into vagal and spinal components. Nerve fibers of vagal and spinal afferents, being mostly unmyelinated C-fibers with only a small percentage A-δ fibers, have endings in every layer of the gut wall yet there remains vast differences between both the types of innervation. Vagal nerves for example, are mostly centered around the more proximal regions of the GI tract such as the small intestine, stomach, and oesophagus, although there is some overlap with the spinal innervation along the small intestines and proximal colon. The most notable difference is that the vagal innervation plays a smaller role in nociception, containing mostly low-threshold nerve fibres responding to non-noxious stimuli involved in nausea and satiety (Sugiura, 2005, Javid, 2013; Kentish, 2015).

1.9.2. Spinal innervation

Spinal afferents, in contrast to vagal afferents, display low or high-thresholds to mechanical stimuli, and are composed of splanchnic and pelvic nerves. Splanchnic nerves innervate the entire GI tract and as such represent a major pathway for the transmission of nociceptive input from the bowel. $TRPV_1$, a prominent ion channel associated with nociceptors and visceral pain has been shown to be present on over 80% of splanchnic afferents (Christianson, 2006; Akbar, 2008). Part of the sympathetic nervous system, they have an approximate ratio of 3:1 for afferents:efferents. Of these afferents, approximately 50% have their endings in the mesentery (Brierley, 2004, 2005). Spinal afferents have their cell bodies in the DRG, with the splanchnic nerve passing through the pre-vertebral ganglia (inferior mesenteric, superior mesenteric, and celiac ganglia), before reaching the

spinal cord where they enter the thoracolumbar and lumbosacral levels of the spinal cord (T10-L2 in rodents) (Christianson, 2006).

The pelvic neurons form part of the parasympathetic nervous system and innervate structures lying within the pelvic region such as the rectum, bladder and reproductive organs, through the hypogastric plexus and pudendal nerves.

In mice, the sympathetic branch has its cell body in the thoracolumbar DRG but the more predominant parasympathetic branch have their cell bodies in the lumbosacral DRG and converge onto spinal neurons in the L6-S2 regions of the spinal cord.

The pelvic nerves have endings within the serosal, mucosal, and muscular layers, and also contain muscular/mucosal afferents. Similar to spinal innervation, the visceral nerve fibers produce poorly localised pain, often termed referred pain, and Minagawa and colleagues found that pelvic nerve C-fibers could innervate both the bladder and colorectal regions with 'convergent fibers' which compound the effects of poor localisation of pain (Minagawa, 2013).

Pelvic neurons are mostly low-threshold fibers which can produce larger responses for a more maintained stimulus than the splanchnic afferents, although high-threshold nociceptors are also found (Brierley, 2008). As the splanchnic afferents contain only around 10% stretch-sensitive fibers compared with 40% found in pelvic innervation, it suggests that the pelvic fibers are more important in noxious stretch and distension (Brierley, 2004; Feng, 2011). The pelvic neurons are hugely important in stretch responses and CRD studies have shown that ligation of spinal afferents will not effect CRD avoidance behaviour in rodents but pelvic nerve ligation will reduce it considerably (Feng, 2010; Kyloh, 2011). In this regard, splanchnic nerves are more involved in noxious stimuli after inflammation providing a greater distinction between the two GI branches on sensory nerves (Traub, 2000). This is not to say however that the pelvic nerves have no involvement in inflammatory pain. The high-threshold afferents will respond to inflammatory mediators such as BK although on a comparatively reduced basis compared with the splanchnic high-threshold afferents

(11% compared with 66% of afferents, respectively) (Coldwell, 2007). Evidence for this also comes from rat TNBS models which show an increase in CGRP-positive DRG neurons innervating the L1 and S1 spinal regions from the bladder, and muscular-mucosal pelvic afferents also exhibit hypersensitivity to CRD after colorectal inflammation (Qiao & Grider, 2007; Feng, 2012).

Pelvic neurons also transmit similar signals as the vagal afferents, such as physiological sensation and have been shown to be involved in acute pain (Brierley, 2005; Blackshaw, 2007).

1.9.3. Primary afferent subtypes in the colon

Over the past decade, the literature has described extrinsic primary afferent endings in each layer of the colon and have characterised their responses to a number of mechanical and chemical stimuli:

(i) Mucosal layer afferents are widely regarded as low-threshold fibers which respond to light stroking with Von Frey hairs but are insensitive to stretch or distension and can be activated by 5-hydroxytryptamine 3 (5-HT_3) agonists, suggesting that they play a role in nutrient sensing within the gut (Brierley, 2004). Mucosal CGRP-positive neurons expressing $TRPM_8$ have been linked to inflammatory processes within colitis through CGRP release in response to $TRPM_8$ activation from inflammatory mediators (de Jong, 2015). This CGRP suppresses pro-inflammatory cytokines and reduces local inflammation suggesting that $TRPM_8$-positive mucosal neurons may have additional roles in health and disease.

(ii) Muscular layer afferents in both the longitudinal and circular muscle respond to stretch and colorectal distensions, and are thought to be responsible for transmitting information based on movement through the bowel.

(iii) Muscular-mucosal afferents are low-threshold fibers that respond to distension combining properties of both types of afferents and they are common in pelvic innervation comprising of approximately 25% of afferents (Brierley, 2004; Feng, 2010; Zagorodnyuk, 2012). These low-

threshold fibers have been suggested to be activated during colonic migrating motor complexes (painful contractions) in IBS (Zagorodnyuk, 2012; Chey, 2001).

(iv) Serosal layer and mesenteric afferents respond to both mechanical Von Frey hair probing and noxious chemicals and have previously been grouped together as 'vascular' afferents (Brookes, 2013). They have endings on or in close proximity to blood vessels and are not observed within the vagal innervation. They are the major type of nociceptor in the colon and are most likely involved in abdominal pain via neuro-immune interactions during disease, and their activation has been specifically linked with inflammatory pain (Brookes, 2013; Brierley, 2004; Hughes, 2009; Brierley, 2008; Wang, 2005). For example, inflammatory mediators such as 5-HT and BK will elicit robust responses in 58% and 66% of splanchnic serosal afferents, respectively (Coldwell, 2007). Several ion channels have been shown to be expressed on serosal afferent endings such as $TRPV_1$, which is well-established as an ion channel linked with nociception (Trevisani, 2002; Zhang, 2005; Yu, 2008; Lapointe, 2015). $TRPM_8$-positive serosal afferents have also been identified in the reduction of chemical and mechanical sensitivity (Harrington, 2011). The $TRPM_8$-positive serosal afferents are co-expressed and possibly coupled to other TRP channels such as $TRPV_1$ and $TRPA_1$ (Harrington, 2011). $TRPV_4$-positive serosal afferents co-expressed with CGRP in IBD patients are responsible for enhanced mechanical sensitivity within inflammatory environments (Brierley, 2008).

Based on mechanical responses 3 distinctive subtypes have emerged: *wide-dynamic range fibers*, which respond to a mixture of low and high levels of mechanical stimuli consisting of A-δ fibers (2-6µm diameter; 12-30m/second conduction velocity) and C-fibers (0.4-1.2µm diameter; 0.5-2m/second conduction velocity); *high-threshold fibers*, with characteristically low resting levels of activity but respond to noxious levels of stretch and distension; *silent nociceptors*, which appear to develop mechanical sensitivity after exposure to inflammatory mediators (Schmidt, 1995). In

addition to this, approximately 25% of splanchnic nerves are mechanically insensitive afferents (M.I.A) (Brierley, 2004; Feng, 2011). The A-β myelinated fiber is also found within spinal innervation although they do not contribute to pain processing. These fibers are typically less than 10μm in diameter and have a very fast conduction velocity of between 30-100m/second, which are used to transmit signals relating to touch.

1.9.4. Vagal innervation

Vagal neurons represent the largest sensory nerve in the human body, with approximately 80% of fibers being afferents (Agostini, 1957; Leek, 1977; Jänig, 1996). The entire gut with the exception of the transverse colon and distal regions are innervated by the vagus. It typically transmits information regarding carbohydrate levels in the GI tract, mechanical distortion and other forces applied to the mucosa, and the presence of bacterial products (Li, 1984; McCaig, 1987; Borovikova, 2000b). The vagal primary afferents have their cell bodies in the inferior vagal ganglia (the nodose ganglia in rodents) synapsing in the brainstem with second order neurons ending in multiple regions of the cortex, most notably the anterior cingulate cortex, amygdala, thalamus, somatosensory cortex, and the anterior insula (Odekunle, 1985; Almeida, 2004; Klarer, 2014).

Within the vagal innervation sub-classes of neurons exist, such as the intraganglionic laminar endings (IGLE's), which were first discovered in oesophagus tissue in 1946, and are situated between the longitudinal and circular muscle (Nonidez, 1946). Many IGLE's have since been found in the proximal GI tract and are known to have regional variability even within the same organ (Andrews, 1980). Early animal studies identified that the IGLE's respond to distension and contraction, perhaps signalling gastric distension after eating (Blackshaw, 1987; Fox, 2000). Intramuscular arrays, another class of neurons in the vagus, project to the nodose ganglia from muscular layers. They are thought to play a role as tension receptors, largely because the highest density of endings have been observed around sphincter regions of the stomach (Wang, 2000). The

vagal mucosal afferents in the intestines are polymodal and work to detect chemical stimuli and although insensitive to direct stretch they do respond to light stroking (Brierley, 2004). As the endings extend through the submucosal layers to the lamina propria and the parenchyma of the mucosal villi, they seem to be ideally positioned to detect mediators released from enteroendocrine cells for nutrient sensing in the gut. Some studies also suggest that mucosal afferents detect changes in osmolarity and pH and may be involved in controlling gastric emptying rates (Powley, 2011; Becker, 1983).

Vagal innervation may also play a small role in IBD pain as vagal nerve stimulation has been shown to be analgesic, possibly due to cross-talk between the vagus and anti-nociceptive actions of the endogenous opioid system in the descending pain pathway (Ghia, 2006; Gschossmann, 2002; Borovikova, 2000a).

Figure 2. The anatomy of the intestinal wall

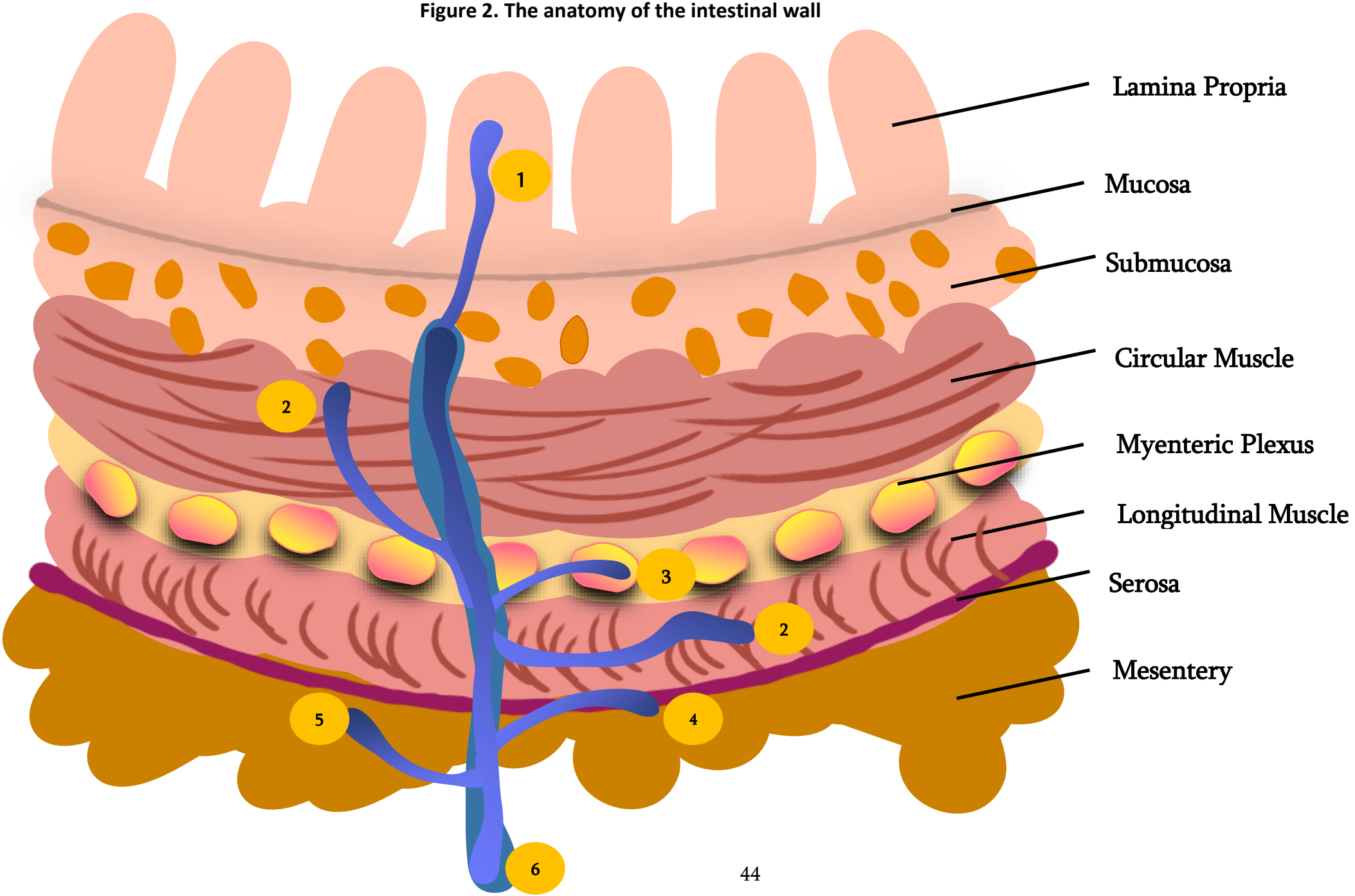


Figure 2. The anatomy of intestinal wall. 1. The mucosal nerves. **2**. The intramuscular arrays (vagus nerves only). **3**. Intraganglionic laminar endings (vagus and pelvic nerves only). **4**. Serosal nerves which were used in this study for electrophysiological recordings. **5**. Mesenteric nerves. **6**. Mesenteric blood vessel.

1.10. Neuronal excitability

Chronic abdominal pain is thought to be the result of a combination of peripheral and central mechanisms. Changes to the gut environment can lead to an overactive nervous system and leave the patient susceptible to chronic pain. In inflammatory disease, this may be the result of adaptive processes in the nociceptor leading to changes in excitability due to a reduced threshold level prior to action potential generation. The threshold for activation of sensory neurons is determined by a delicate balance of voltage-gated sodium, potassium, and calcium channels, preceded by a heterogenous array of GPCR's coupled to ion channels such as TRP and ASIC channels.

For example, potassium channels such as TREK channels (TWIK-related potassium channels) are expressed on small and medium sized DRG's and often co-localise with $TRPV_1$ where their increased expression has been associated with features of neuropathic pain (Maingret, 2000; Talley, 2001). TREK channels are mechano-sensitive potassium channels and TREK-2 channels also regulate neuronal excitability on thermosensitive $TRPV_1$-positive C fibers whereas TREK-1 activation can lead to hyperpolarization of DRG membranes to reduce nociceptor activity.

Ion channel transducers expressed on nociceptors serve to produce the inward currents which then lead to generator potentials which activate voltage-gated sodium channels (Nav). There are 3 distinct Nav channels (1.7, 1.8, 1.9) which are expressed on nociceptors. The absence of the TTX-sensitive $Nav_{1.7}$ has been shown to lead to a congenital insensitivity to pain in humans and rodents, and *in vitro* imaging of mouse DRG's has also revealed reduced action potentials when this channel is lost (Gingras, 2014). The TTX-resistant $Nav_{1.8}$ and $Nav_{1.9}$ channels are responsible for firing characteristics and nociceptor thresholds, respectively. Voltage-gated sodium channels represent a favourable target for pharmacological intervention within visceral pain, for example, the $Nav_{1.8}$ channel has been reported to be upregulated as a result of chronic inflammation in mice, through an increased translocation to the afferent membrane (King, 2009). This change would effect

the neuronal excitability and has been shown to be responsible for altering the repetitive firing characteristics of the afferent which is apparent during ongoing afferent activation such as that during chronic inflammatory disease, and interestingly, $Nav_{1.8}$ gain-of-function mutations have been identified in painful peripheral neuropathy (Rush, 1998).

$Nav_{1.9}$ is predominantly expressed on small diameter neurons providing a large persistent sodium current and is heavily implicated in inflammatory pain (Hockley, 2014). The $Nav_{1.9}$ voltage dependence of activation and inactivation lies very close to the resting membrane potential which supports the hypothesis that $Nav_{1.9}$ regulated neuronal excitability of peripheral nociceptors by modulating the resting membrane potential (Baker, 2003). This has also been well demonstrated in a recent study where $Nav_{1.9}$ mutations caused a dampening in the excitability of DRG neurons due to a deficiency in $Nav_{1.9}$ functioning to depolarise the membrane following subthreshold excitation, and this mimics clinical observations where pain sensitivity is lost (Huang, 2017).

IBS is recognised as presenting visceral hypersensitvity as a key feature. IBS patients, although do not initially appear to have signs of ongoing inflammation, nevertheless demonstrate elevated pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα, predominantly in adult IBS-D patients (Hughes, 2013). Pro-inflammatory mediators such as kinins, prostaglandins, purines, NGF, and protons, which can produce post-translational adaptions in transducer channels and Nav channels via intracellular kinases lead to changes in neuronal excitability through adapted membrane thresholds and modified kinetic properties of nociceptors. The pro-inflammatory cytokine IL-1β is a potent activator of nociceptors where it is thought to recruit TTX-sensitive voltage-gated sodium channels, such as $Nav_{1.7}$, through IL-$1R_1$ activation (Hughes, 2013; Binshtok, 2008). This IL-1β also increases neuronal excitability via p38 MAP kinase by enhancing $Nav_{1.8}$ and $Nav_{1.9}$ currents (Binshtok, 2008). This excitability occurs due to the $Nav_{1.8}$ influence which is responsible for the majority of the inward current during the upstroke in action potentials in DRG's.

T-type calcium channels expressed on primary sensory neurons are also key regulators of neuronal excitability where they are highly sensitive to low-level depolarisation. They mostly regulate sub-threshold excitability within the CNS but have been observed in small diameter DRG's. They enhance the excitability of neurons by lowering the membrane threshold to contribute to calcium entry and have been implicated in

inflammatory pain and neuropathic pain.

Chronic pain states have described changes in the peripheral nociceptors which result in a lower threshold for activation. Long term sensitisation of GI afferents, including MIA's, are understood to contribute to hypersensitivity of the bowel, and the peripheral environment surrounding sensory afferents which results in nociceptor adaptions, appear to be one of the most important factors (Feng, 2012). For example in both IBS (including post-infectious IBS) and IBD increased $TRPV_1$ expression has been observed and correlates with patient pain reporting (Akbar, 2008; Akbar, 2010; Balemans, 2017). When PI-IBS was modelled in mice, the DRG's became more sensitive to low doses of capsaicin, which was repeated in the submucosal neurons from patient biopsies (Balemans, 2017).

The authors suggested that the increased $TRPV_1$ expression would result in greater neuronal activation via a signal transduction mechanism involving the histamine (H_1) receptor. In support of this finding, histamine has previously been associated with post-inflammatory visceral hypersensitivity where the H_1 receptor is coupled to $TRPV_4$. (Deiteren, 2014). The release of histamine and its resulting binding to the GPCR H_1 receptor on afferent endings leads to the phosphorylation of $TRPV_4$ channels via PLC and PKA activation, and also upregulates translocation of $TRPV_4$ channels to the membrane (Cenac, 2008). In a similar manner, 5-HT, acting predominantly through the 5-HT_3 receptor, has been shown to stimulate vagal and intrinsic afferents, activating $TRPV_4$ channels via PLC, PKA, and PLA_2, creating arachidonic acid metabolites which can act as endogenous $TRPV_4$ agonists although 5-HT can also activate Nav channels (Cenac, 2008; Cremon, 2011; Watanbe, 2003). Chronic exposure of mast cell mediators likely result in peripheral modulation and an overall increase in the excitability of the nociceptor, and convincingly, mast cell proximity to sensory nerves does correlate to increased pain scores in patients (Di Nardo, 2014).

Proteases have also consistently been shown to increase afferent firing and gut inflammation leads to an increased expression of PAR_2 (Reed, 2003; Kim, 2003; Lohman, 2012). Mast cell tryptase, cathepsin S, and neutrophil elastase, for example, signal through the GPCR PAR_2, and PAR_2-specific agonists can induce visceral hypersensitivity in rats (Coelho, 2002; Kawabata, 2001). Activation of PAR_2 leads to the downstream activation of $TRPV_1$ and $TRPV_4$ channels through PLC-mediated intracellular pathways and chronic exposure to these proteases is therefore associated with sensitisation from coupling of GPCR's to signal transducer

channels (Zhao, 2015; Poole, 2013; Grant, 2007; Grace, 2014). Furthermore, PAR_2 activation can lead to the release of SP and CGRP which can potentiate $TRPV_1$ and $TRPV_4$ channels (Niizeki, 1997; Engel, 2011).

$TRPV_4$ is a member of the transient receptor potential vanilloid family of ion channels. First discovered in 2000 it is now known to be a non-selective 6-transmembrane cation channel with a slight preference for calcium ions where it is activated at temperatures of 27-35°C, lipid derivatives, and noxious mechanical stimuli. The N-terminus consists of 3 ankyrin repeat domains which aid its function as a mechanical-sensitive channel, whereas the C-terminus is involved in calcium ion exchange through PDZ and calmodulin domains. $TRPV_4$ is widely expressed endogenously, and since its expression was observed in retrogradely-labeled vagal nodose ganglia neurons in 2004 a host of laboratories have shown its expression in mouse CGRP-positive colonic neurons, intestinal epithelial cells, human T-cells, human mast cells, renal tissue, skin, and the cornea (Brierley, 2008; Zhang, 2004; Sipe, 2008; Cenac, 2008; Majhi, 2015; Kim, 2010; Tian, 2004; Pan, 2008; Chung, 2011). Within normal physiological conditions, $TRPV_4$ has functions involved in oedema and granulocyte infiltration during normal tissue injury whose effects have been studied using hypotonic solutions and $TRPV_4$-specific agonists, where effects were reduced in mice with the $TRPV_4$ gene deleted (Vergnolle, 2010). However, $TRPV_4$ has been widely studied for its involvement in many types of pain. There is evidence to suggest that it is involved in somatic pain where the channel serves as a high-threshold mechanoreceptor in addition to mediating allodynia and hyperalgesia (Suzuki, 2003; Sipe, 2008; Cenac, 2008; Vergnolle, 2010; Alessandri-Haber, 2004; Alessandri-Haber, 2005). $TRPV_4$ has also been associated with mechanical hyperalgesia in pancreatitis and sensory neurons undergoing chronic constriction injury in mice (Zhang, 2008; Ceppa, 2010). The $TRPV_4$ agonist 4-alpha-phorbol-12-12-idecanoate (4αPDD) is able to induce strong CNS activation (measured by c-fos expression) in WT mice but not in $TRPV_4^{-/-}$ mice, and interestingly, $TRPV_4^{-/-}$ mice exhibit reduced pain behaviours in a model of pancreatitis compared with WT mice (Ceppa, 2010).

With the discovery that $TRPV_4$ helps to mediate mechanical pain, the increased mRNA and protein of $TRPV_4$ in the colon of humans and rodents following inflammation added to the growing evidence that it could be targeted for visceral pain (Grant, 2007; Cenac, 2008; Ceppa, 2010; Brierley, 2008; Fichna, 2012). Mechanical pain studies using $TRPV_4^{-/-}$ mice have been able to show a 50% reduction in colonic afferent firing from baseline VFh probing responses (Meuller-Tribbensee, 2015; Sipe, 2008; Brierley, 2008).

The lipid metabolite and TRPV4 agonist EET potentiates VFh proved responses in mouse colonic afferents and this was shown to be abolished in TRPV4-/- mice (Brierley, 2008). In contrast to this, VMR using noxious CRD have observed similar levels of afferent responses in $TRPV_4^{+/+}$ and $TRPV_4^{-/-}$ mice, which the authors suggested was due to the limitations of CRD to proficiently activate serosal layer nociceptors although this claim is yet to be fully substantiated (Sipe, 2008).

Due to the channels apparent involvement in mechanical hyperalgesia much focus on the mechanisms of its role have led to a better understanding of its activation. One key pathway seems to be dependent on PAR_2 activation. Our current understanding is that as PAR_2 is cleaved, potentially by proteases such as neutrophil elastase, $TRPV_4$ is activated via intracellular signalling pathways utilising adenylyl cyclase, PKA, and arachidonic acid metabolites (Grant, 2007; Poole, 2013; Grace, 2014; Sipe, 2008; Zhao, 2015; Cenac, 2015; Sisignano, 2012). This leads to membrane depolarisation and the release of SP and CGRP which in turn can contribute to pain pathways (Grant, 2007). SP can induce epithelial release of IL-8, promoting neutrophil recruitment, and also directly lead to the sensitisation of $TRPV_1$-positive neruons, thereby resulting in the release of further SP and CGRP, promoting further inflammation (Zhao, 2002; Engel, 2012). In support of the finding that PAR_2 couples to $TRPV_4$ on peripheral nociceptor membranes, $TRPV_4^{+/+}$ mice but not $TRPV_4^{-/-}$ mice showed a robust response to PAR_2 agonists (Sipe, 2008). As a further display of this mechanism, PAR_2 agonists will also potentiate CRD in $TRPV_4^{+/+}$ mice but not in $TRPV_4^{-/-}$mice (sipe, 2008). $TRPV_4$ -mediated somatic pain in mice is potentiated in the presence of inflammatory mediators suggesting a role in inflammatory pain within the somatic system also (Alessandri-Haber, 2005). This leading role, and our current understanding of its cellular mechanisms mean that it has great potential as a target for visceral pain therapies, and the aim of this study was to understand if this channel could play a major role in afferent activation from the colonic environment.

1.11. The ascending and descending pain pathway

The nociceptive pathway extends beyond peripheral influences and central sensitising effects have been suggested to contribute to chronic pain. The primary afferents from the colon enter and synapse with second order afferents (excluding interneurons) at the thoracolumbar and lumbosacral levels within the spinal cord.

The neurons travel through laminae I, II, V, and X, where several synapse and ultimately lead to the cortex. The spinothalamic tract has synapses within the hypothalamus, via third order neurons, projects to areas of the limbic system such as the amygdala, the ACC, and medial thalamus, along with the LC and PAG. The resulting neuronal projections are responsible for emotional, autonomic, and behavioural responses. The spinothalamic tract is the major route for nociception and pain, which eventually synapse in the thalamus, specifically, the ventromedial posterior nucleus (VMpo), ventral posteromedial nucleus (VPM), and ventral posterolateral nucleus (VPL) (see figure 3).

The descending pain pathway is responsible for modulating the pain signals towards the brain. The ACC, which has a dual function in projecting pain signals and reducing them, has connections to the PAG, RVM, and raphé magnus, which all contribute to the release of endogenous opioids such as β-endorphin and enkephalin, in addition to noradrenaline, dopamine, and 5-HT, which have an intricate involvement in propagating and diminishing nociceptive signals (Schweinhardt & Bushnell, 2010). A study regarding gender differences in IBS patients found that a reduction in the contribution of the descending pathway was common in IBS patients compared with healthy volunteers (Naliboff, 2003). The authors also found that the dorsal pons and amygdala were activated to a greater extent in males, whereas the ACC contribution was greater in females, suggesting that activation of facilitatory circuits may reduce the impact of descending modulation, an observation that was later replicated in UC (Naliboff, 2003; Mayer, 2005).

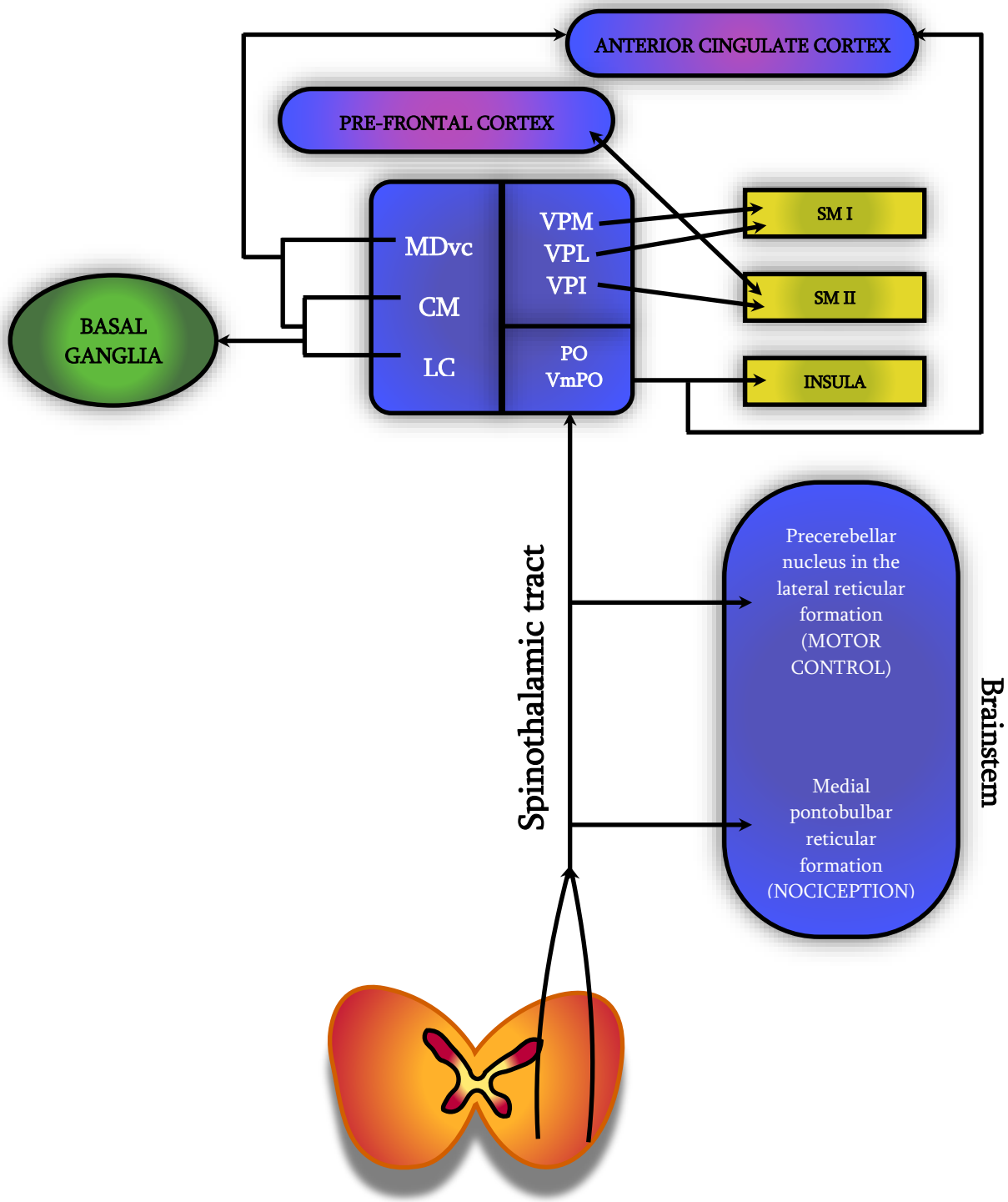


Figure 3. The ascending pain pathway.

The neuronal projections from the spinothalamic tract originate form the spinal cord dorsal horn. Synapsing within various regions in the cortex and processed by the thalamus and somatosensory regions. LC, locus coeruleus; CM, centromedian nucleus; MDvc, medial dorsal ventral caudal nucleus; VmPO, ventromedial

posterior nucleus; PO, posterior nucleus; VPI, ventral posterior inferior nucleus; VPL, ventral posterolateral nucleus; VPM, ventral posteromedial nucleus; SM, supplementary motor area.

1.12. Visceral hypersensitivity

Our current understanding in adult FGID's such as IBS is that visceral hypersensitivity is a key feature responsible for chronic abdominal pain. In 2001, several studies were published utilising electronic barostat techniques in patients. Ginkel and colleagues (2001) found that visceral hypersensitivity was a key feature in IBS paediatric patients but not in FAPS patients. In contrast to this, Di Lorenzo (2001) found that children with RAP did present with visceral hypersensitivity. The different observations are difficult to decipher due to limited detailed methodologies from Di Lorenzo and because the two pain phenotypes are clinically very similar. It does however, highlight that lower thresholds for activations on visceral nociceptors are likely a major contributor to increased visceral pain in patients. This hypersensitivity has been suggested to be the result of repetitive stimulation from endogenous mediators and previous studies have implicated mast cell mediators in this process (Di Nardo, 2014, Klooker, 2010). Mast cell degranulation based therapies have already resulted in reduced abdominal pain in IBS patients suggesting a promising therapeutic pathway for the future (Klooker, 2010).

1.13. Mast cell infiltration

Despite the lack of organic or structural changes in the gut of FGID patients, there is a growing consensus of an altered gut environment. One key finding that has been replicated in several studies is the observation detailing increased mast cell infiltration in the mucosa of biopsies from adult IBS patients (Barbara, 2007). The supernatants of IBS biopsies were analysed for key mast cell mediators and found to have significantly higher levels of histamine, tryptase, and PGE_2, when compared to healthy controls (Henderson, 2012; Taylor, 2010; Di Nardo, 2014; Buhner, 2009; Dat, 2015). The effects of IBS supernatants from adult mucosal biopsies on rodent intestinal nerves induces afferent firing and results in hypersensitivity to blunt probing and colorectal distension (Barbara, 2007; Balemans, 2017; Crouzet, 2013). This afferent activation was shown to be the result of mast cell mediators histamine and tryptase. Buhner and colleagues (2009) have published

similar data in human submucous neurons whereby IBS supernatant added to the nerves directly caused a robust activation predominantly from tryptase release.

Human mast cells have also been shown to release high quantities of 5-HT and studies have correlated local 5-HT concentrations with severity of pain symptoms in IBS-C and IBS-D patients (Fox, 1985; Barbara, 2007; Taylor, 2010; Cremon, 2011). Furthermore, along with increased levels of 5-HT, decreases in SERT mRNA in children with IBS suggests that a combination of elevated 5-HT and a diminished 5-HT breakdown by SERT is responsible for dysregulation of 5-HT which contributes to pain (Coates, 2004; Faure, 2010). Barbara and colleagues (2004) were able to show that mast cell proximity to afferent nerves results in afferent activation and a recent study correlated paediatric abdominal pain scores with mast cell proximity to nerves that may explain some origins of abdominal pain in IBS (Di Nardo, 2014).

1.14. Potential pain mediators from mast cells

As the breakdown of mast cell mediators such as histamine and 5-HT can occur rapidly this study used qPCR to assess the mRNA expression of the enzymes involved in the production of both histamine and 5-HT. To this end, histidine decarboxylase and tryptophan hydroxylase were measured in this study. Figure 4 below demonstrates the enzymatic pathway involved in the production of both histamine and 5-HT.

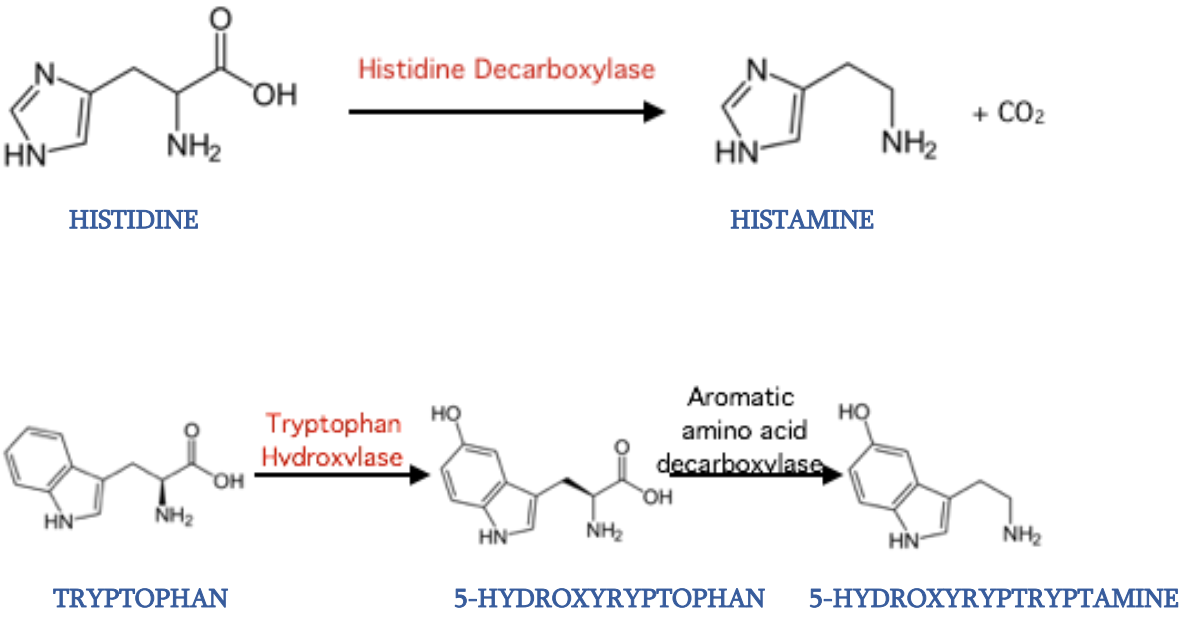


Figure 4. Enzymes important in the production of histamine and serotonin.

(Above). Histamine is produced from histidine by histidine decarboxylase. **(Below)**. Serotonin (5-hydroxytryptamine) is produced from the conversion of tryptophan to 5-hydroxytryptophan by tryptophan hydroxylase, which is then converted to serotonin by the enzyme aromatic amino acid decarboxylase. Tryptophan hydroxylase is the rate-limiting enzyme in serotonin synthesis.

Histamine is one of the many inflammatory mediators released from mast cells and it has been shown to be elevated in FGID's (Buhner, 2009, Nasser, 2014). Histamine has four known G-protein coupled receptors (1-4), and are responsible for smooth muscle contraction (H_1), gastric secretion (H_2), vasodilation (H_3) and increases in vascular permeability (H_4), and all play a role in inflammation. Histamine is produced by the breakdown of histidine by histidine decarboxylase and levels of this enzyme vary depending on the requirement for histamine at inflammatory sites meaning that levels of expression of this enzyme could relate to altered levels of histamine (Alcañiz, 2013) (see figure 4).

Research investigating the sensitising effects of histamine have shown that thermal and mechanical hyperalgesia can be attenuated using H_1 and H_2 receptor antagonists (Zuo, 2003). This observation was also supported by rodent models of tissue injury using the formalin test, where H_1/H_2 receptor antagonists reduced nociceptive responses, and in both studies the H_2 receptor had the more dominant involvement (Mobarekeh, 2011). This study also implicated histamine in neurogenic inflammatory processes suggesting that histamine results in the release of CGRP from afferent endings which then works synergistically to increase local mast cell degranulation. Further contributions of histamine on the H_2 receptor can result in the up-regulation of $Nav_{1.8}$ in DRG's and blocking this receptor function can limit thermal and mechanical allodynia (Yue, 2014). In addition, experiments adding histamine directly to sensory afferents in mesenteric tissue have shown robust levels of activation demonstrating that direct pro-nociceptive actions also result from exposure to histamine (Kreis, 1998). Furthermore, histamine has also been shown to sensitise $TRPV_1$ on visceral nociceptors through the H_1 receptor and this mechanism has been suggested to be responsible for visceral hypersensitivity in IBS (Wouters, 2016). The histamine receptors H_3 and H_4 have also been linked with roles in nociception and pain. When a H_3/H_4 receptor antagonist was used in mice a sharp reduction in

thermal pain behaviour was observed which the authors suggest was driven by an inhibition of mast cell signalling (Chatterjea, 2012). As the H_3 receptor is located in the CNS and the H_4 receptor is peripherally available, these effects were likely the result of H_4 receptor-driven pathways. Single-unit electrophysiological recordings have identified histamine-responsive neurons in the superficial laminae of the dorsal horn, a region involved in nociceptive transmission and as such, when H_3 receptor antagonists have been used, they have been shown to reduce allodynia and hyperalgesia in neuropathic and inflammatory pain models (Akiyama,2014; Medhurst, 2008; Hsieh, 2010)

Serotonin (5-HT), released from enterochromaffin cells (EC) regulates sensory, motor and secretory functions in the GI tract, and it has been suggested that alterations in the levels of 5-HT may contribute to hypersensitivity in the gut. Cremon *et al.* (2011) observed increases in 5-HT-positive cells in mucosal biopsies of IBS patients and a 10-fold increase in 5-HT levels when compared to healthy controls, and this increase was linked to abdominal pain through 5-HT_3 receptors expressed on both extrinsic and intrinsic primary afferent neurons. 5-HT is known to play a role in inflammation and a study from Bischoff's laboratory in 2009 showed how TNBS mice lacking the serotonin transporter developed much more severe colitis while noting that the relative levels of 5-HT or EC cells did not change compared with wild type mice confirming that the effects were due to the decreased clearance of 5-HT resulting in increased receptor interaction (Bischoff, 2009). Tryptophan hydroxylase, the rate-limiting enzyme used in the formation of 5-HT (figure 10), has been shown to be expressed in areas of high 5-HT release, such as EC cells, the spleen, brainstem and neurons of the myenteric plexus (Ghia, 2009). DSS and TNBS models using tryptophan hydroxylase knockout mice resulted in only mild colitis reflecting the reduced availability of 5-HT once the tryptophan hydroxylase is removed (Ghia, 2009). The mice lacking this enzyme also showed lower levels of TNFα, IL-6 and IL-1β, reflecting the decreased inflammatory environment. These observations suggest that tryptophan hydroxylase may be a good marker for 5-HT involvement in the gut.

Belonging to the family of serine proteases, tryptase is released from mast cells at sites of inflammation and makes up 35% of the total protein content within mast cells (Schwartz, 1981). Tryptase has been shown to be raised in IBS patients due to mast cell infiltration within the gut (Di Nardo, 2014; Buhner, 2009; Cenac, 2007; Liang, 2016). Studies of visceral inflammatory pain in humans have revealed tryptase as an important

mediators in nociception. A 2014 study by Roman *et al*. firmly established tryptase as a key mediator in inflammatory pain in prostatitis patients and also found that PAR_2, a GPCR for tryptase could be blocked to reduce pain (Roman, 2014). A study in a similar cohort of patients one year later identified tryptase as also being responsible for mechanical hypersensitivity which has been linked with $TRPA_1$ involvement (Schwartz, 2015). These studies contribute to the observation that tryptase can directly cause hyperexcitability when applied to submucosal neurons of the gut and that PAR_2 agonists can lead to visceral hyperalgesia in rat *in vivo* experiments, possibly through additional spinal cord neuronal activation from PAR_2 signalling at the peripheral afferent (Reed, 2003; Coelho, 2002). Tryptase has also been implicated in neurogenic inflammatory processes where PAR_2 activation can lead to CGRP and SP release from afferent endings, which have been linked to abdominal pain severity in IBS (Liang, 2016).

1.15. IBD pain

1.15.1. IBD pain research

Abdominal pain in IBD is a leading source of morbidity amongst patients yet the basic scientific research does not reflect this. Consequently, our understanding of the mechanisms behind abdominal pain in IBD is poor. Studying the inflammatory process involved in IBD has offered a pragmatic approach to identifying key features such as pro-inflammatory cytokines, and several (such as IL-6 and IL-1β) have since been shown to have the ability to robustly activate visceral afferents (Schrieber, 1995; Reddy, 2007; Arranz, 2009; Henderson, 2012; Hughes, 2013). However, this is likely to not represent the entire mechanism of abdominal pain. For instance, IBD patients with resolved inflammation still report periods of significant abdominal pain (Coates, 2013; Zeitz, 2016). Several studies also implicate immune cell mediators such as 5-HT and mast cell tryptase in visceral pain in the GI tract (Matsumoto, 2012; Cenac, 2007). Even so, it is unlikely that cytokines and immune cell mediators are capable of eliciting an action potential by themselves and coupling to specific ion channels such as TRP channels has been observed (Matsumoto, 2012; Lapointe, 2015). TNBS models have confirmed previous *in vitro* reports suggesting $TRPV_1$ coupling to 5-HT receptors, along with PAR_2 (Matsumoto, 2012; Amadesi, 2006). Both animal models have consistently shown an up-regulation of $TRPV_1$ and demonstrated its responsibility for visceral sensitisation (Hughes, 2009; Miranda, 2007). Despite these

observations, animal models still only focus on the post-inflammatory environment and as a result, reflect changes in a state of inflammatory remission which may not truly represent the actively inflamed gut in IBD patients. Understanding the changes within nociceptors and the local inflammatory environment during inflammation is therefore compromised.

1.15.2. Human studies

Few patient-led studies for IBD pain exist although a number of pro-nociceptive mediators have been identified as candidates for pain research. ATP is associated with afferent activation and is released in high concentrations from epithelial cells during colonic distension and the P_2X_3 receptor has been observed in rodent sensory nerves within the gut and been linked to hypersensitivity (Hockley, 2016; Shinoda, 2009). Human expression studies of the P_2X_3 receptor have shown elevated levels within the colon of IBD patients and so taken together there is strong evidence for its role within IBD pain (Yiangou, 2001). Serine proteases such as cathepsin's have been shown to be increased in IBS and UC patients where they act to both promote and inhibit nociception within sensory neurons of the colon (Annaházi, 2009). In a similar manner, IBS patients demonstrate elevated levels of mast cell tryptase, where its ability to directly activate sensory neurons has been demonstrated (Reed, 2003). Pragmatically, IBD studies have also reported increased levels of tryptase (Raithel, 2001).

This current study uses biopsies from IBD patients with chronic abdominal pain to understand the colonic environmental changes that lead to a pro-nociceptive gut which then has the ability to elicit afferent firing. This study is the first of its kind to combine IBD patient biopsies of ongoing disease with visceral afferent responses to understand the changes within the GI environment and the mediators that result in nociception.

1.16. The immune response

1.16.1. Innate immune system

The innate immune system is responsible for the immediate response to pathogens and unlike adaptive immunity it is non-specific and has no lasting memory. It is generally involved in bacterial killing, antigen

presentation, and initiating the further release of pro-inflammatory cytokines. In the gut of IBD patients, the innate immune response is triggered when microbes infiltrate the disrupted epithelial cell barrier in genetically susceptible hosts. There are several important cell types within the innate immune system that work synergistically to provide a rapid response to invading pathogens. Several features of the structure and physiology of the gut provide protection and have a valuable role in the innate immune system and are discussed below.

Epithelial cells provide a physical barrier with tight junctions, adheren junctions, and apical junction complexes between cells, to protect against the luminal contents, also relying on an inner and outer mucous barrier for protection. Within the epithelial barrier goblet cells release gel-forming mucins to form a protective barrier, where the inner layer is sterile due to the release of anti-microbial peptides from paneth cells, specialised to the base of the crypts of the small intestine. The epithelial barrier in UC patients if often reported to be deficient in goblet cell production of mucins which expose the host epithelial barrier to microbes. CD patients have a deficiency in anti-microbial peptides released from the epithelial wall, in particular the β-defensins HBD2, HBD3, and HBD4 (Wehkamp, 2003).

The toll-like receptors and NOD-like receptors are vital first responders which recognise the structural motifs of micro-organisms termed pattern associated molecular patterns (PAMPS), resulting in downstream cytokine release and T-cell activation which can bridge into the adaptive immune response. In the healthy gut, TLR activation results in a tolerance to pathogens and a down-regulation of pattern recognition receptors in order to promote mucosal tissue healing and repair, whereas in IBD the continued immune response results in an increased epithelial barrier permeability and an impaired mucosal healing process triggering IL-8 release from epithelial cells and attracts neutrophils and resident macrophages.

Nucleotide-binding oligomerisation domain-containing protein 1 and 2 (NOD1 and NOD2) are cytosolic proteins within epithelial cells and paneth cells which respond to bacteria infiltration activating NF-κB and MAPK gene transcription for downstream innate and adaptive immune cells and mediators (Barnich, 2005). The NOD proteins are important in maintaining the integrity of the epithelial barrier by contributing to tissue homeostasis thought to be through tonic stimulation by gut microbiota which results in the release of defensins, although the precise mechanisms are not yet fully understood (Rubino, 2012).

In 2001, *NOD2* became the first gene to be associated with CD and remains a strong risk factor to developing the disease (Hugot, 2001). In the absence of NOD2, mouse models show significant epithelial layer damage likely as a result of defects in the recovery of mucosal tissue damage (Kobayashi, 2005). Although important in the development of disease, NOD2 signalling results in downstream release of several pro-inflammatory cytokines and chemokines such as TNFα, IL-6, IL-8, CCL2, CXCL2, which attract neutrophils and monocytes and play a role in driving Th_2 immunity (Rubino, 2012). In general, TLR's are understood to stimulate Th_1 immunity and NOD signalling drives Th_2 immunity through activating dendritic cells, regardless of the fact that Th_2 immunity is classically derived from extracellular pathogens. In addition, a NOD2 variant has also been reported to inhibit the expression of the anti-inflammatory cytokine IL-10 in human monocytes suggesting that NOD2 mutations may also result in compromised immune regulation (Noguchi, 2009).

Dendritic cells reside along the epithelial barrier and reach into the luminal contents to sample the local environment. Once activated they signal through IL-23 to naive $CD4^+$ T-cells which proliferate into Th or T-reg cells to induce the adaptive immune response.

Macrophages are leukocytes responsible for the detection, phagocytosis, destruction of bacteria and dead cells, and the releases of cytokines and proteases which further promote inflammatory cell involvement. Traditionally, they are categorised as either M1 (classical) or M2 (non-classical), where M1 macrophages proliferation from $CD14^+$ monocytes is induced by IFN-ɣ and microbial products resulting in phagocytosis of microbes and the release of pro-inflammatory cytokines such as IL-1β, TNFα, IL-6, IL-8, IL-23, and further INF-ɣ release. The M2 macrophages typically proliferate from $CD14^+/CD16^+$ monocytes induced by IL-4 and IL-13 cytokines and promote anti-inflammatory signalling (Busch-Dienstfertig, 2012). Approximately 90% of macrophages observed in IBD are M1, and active CD also results in reduced M2 activity (Hunter, 2010). Therapies targeting IBD may also indirectly effect the ratio of macrophage subtypes with anti-TNFα therapies reportedly increasing the level of M2 macrophages within the mucosa which may contribute to the treatment effect (Vos, 2011).

Innate lymphoid cells (ILC's) have been studied for a long time, distinctly NK cells, but emerging research focuses on a relatively recent series of 3 subsets (Bull, 1977). Together, ILC's respond rapidly to cytokine and microbial signals to release cytokines which help modulate the adaptive immune response. ILC's are

particularly enriched in tissues such as the skin, lung, and intestinal mucosa and exhibit both anti- and pro-inflammatory characteristics.

Type 1 ILC's express t-bet in response to signals from IL-12. They produce and release TNFα and INF-ɣ in response to intracellular pathogens (Fuchs, 2013). The more widely known example of type 1 ILC's include NK cells, but there are subsets within type 1 ILC's themselves.

Type 2 ILC's respond to signals from IL-25 and IL-33 and elicit an immune response to extracellular parasites. They express the GATA3 transcription factor and when activated produce and release several cytokines such as IL-4, IL-5, IL-9, and IL-13, to encourage an inflammatory response (Moro, 2010; Neill, 2010). Due to the bacteria infiltration associated with IBD from epithelial barrier dysfunction, the type 2 ILC's have been the focus of intense IBD research.

The type 3 ILC's act in a similar manner to Th_{17} cells and respond to cytokine signalling from IL-1β, IL-23 and IL-6, where they produce and release IL-17 and IL-22, particularly in response to extracellular bacteria and fungi (Buonocore, 2010; Cupedo, 2009). Several studies have indicated that IBD patients suffer from a reduced expression of type 3 ILC's and research is currently focused on restoring the potential benefits of these ILC's (Buonocore, 2010; Geremia, 2011).

1.16.2. Adaptive immune system

The innate immune response is a prerequisite for excessive activation of the adaptive response which is the main driver for inflammatory damage in IBD. Unlike the innate response however, adaptive immunity is highly specific and offers long term immunity through B-cell activation. The specificity of the adaptive response comes from the proliferation of Th_0 cells which differentiate based on specific signalling mechanisms (see figure 20 & 21). Th_1 cells were first recognised for their ability to target intracellular pathogens and have been strongly associated with CD where they are induced by elevated mucosal levels of IL-12 and IL-18, and macrophages in CD have been shown to release high concentrations of IL-12 (Monteleone, 1997). The Th_1 cells release various cytokines such as TNFα, IL-2, IL-10, signalling through STAT1 and STAT4 pathways. In addition to releasing TNFα themselves, Th_1 cells also induce the release of TNFα by activating mucosal macrophages, which causes the differentiation of stromal cells into

myofibroblasts, thereby releasing MMP's. The Th_2 cells focus on targeting parasitic invasions, and are crucial in allergic reactions. Several Th_2 cytokines such as IL-5, IL-1β, IL-6, are elevated in UC compared with CD leading researchers to suggest that UC is a Th_2-dominant disease, which has been shown to signal through STAT6 and GATA3 (Rosen, 2011). Although CD and UC present with Th_1- or Th_2-dominant pathologies, the Th_{17} cell type shares common involvement with both diseases. The Th_{17} cells target extracellular pathogens and fungi, and in IBD release cytokines such as IL-17, IL-21, and IL-23, significantly contributing to the pathogenesis of IBD. They differentiate as a result of signals from cytokines such as IL-6, IFN-ɣ, and TGF-β, and the cytokines IL-21 and IL-23 also promote Th_{17} cell proliferation which in turn release more IL-23 in a positive feedback loop. Furthermore, IL-23R polymorphisms have been associated with IBD in genomic studies suggesting that this specific cytokine pathway may be pivotal in IBD (Newman, 2009). IL-21 can stimulate myofibroblasts to produce and release MMP's leading to local tissue destruction, and IL-21 also leads to the attraction of further T-cells at inflammatory sites. The IL-17 released from Th_{17} cells has a significant role in IBD. IL-17 can attract neutrophils to local sites of inflammation which in turn releases cytokines such as IL-8. IL-17 is also central in the up-regulation of nitric oxide and IL-1β, both of which are pro-inflammatory mediators, and stimulates macrophage release of further pro-inflammatory cytokines (Awane, 1999).

T-regulatory cells (Treg) are a subset of $CD4^+$ T-cells that suppress Th_0 proliferation thereby suppressing the immune response (Schevach, 2009). IBD models have established that dysfunction of Treg cells can lead to increases in transcription factors t-bet, STAT1, and NF-kB, potentiating T-cell activation and the adaptive immune response (Ishimaru, 2008).

There are several subsets of Treg cells within multiple locations in the body each with differing characteristics. Natural Treg (nTreg) cells are found within the thymus and secrete IL-10 and TGFβ to have a broad range of suppressive actions on T-cell proliferation, dendritic cell and T-helper cell activity. They become activated predominantly through the IL-2 signalling pathway and mice with IL-2 signalling deficiencies develop spontaneous IBD-like pathology (Setoguchi, 2005). Peripheral Treg (iTreg) cells have similar actions to nTreg cells but express CD4 and $FOXP_3$ markers with an absence of CD25. They develop from $CD4^+$ T-cells during the immune response through TFGβ signalling (Chen, 2003). The tissue-resident

$FOXP_3^+$/$CD25^+$ Treg cells that are a main interest in IBD mucosal tissue are generated from nTreg cells and are highly expressed within GI tissue. The Treg cells negatively regulate T-cell function by signalling through released IL-10, IFN-ɣ, and TFGβ, and have been shown to specifically down-regulate the Th_{17} response in mice via TGFβ (Schevach, 2009; Read, 2006; Harrison, 2015; Nava, 2010). Transfer of Treg cells into IBD mouse models has also demonstrated this inflammatory control whereby IBD-like lesions and mucosal inflammation were reduced, suggesting that Treg cell dysfunction plays a major role not only in the pathogenesis of IBD but also during active disease (Martin, 2004).

Treg cells have been shown to be reduced in IBD patients although some authors reports that the suppressive properties remain active (Maul, 2015). An endogenous inhibitory molecule for Treg function has been identified as a therapeutic target with promising results in animal models (Monteleone, 2001; Fahlén, 2005). Anti-TNFα treatment in IBD patients has been shown to positively effect $FOXP_3^+$/$CD25^+$ Treg frequency and potentiate the suppressive properties which coincide with the inflammatory control through reduced TNFα activity (Boschetti, 2011). Therefore current treatments may enhance Treg actions and contribute to inflammatory control.

Figure 5. The pathogenesis of Crohn's disease

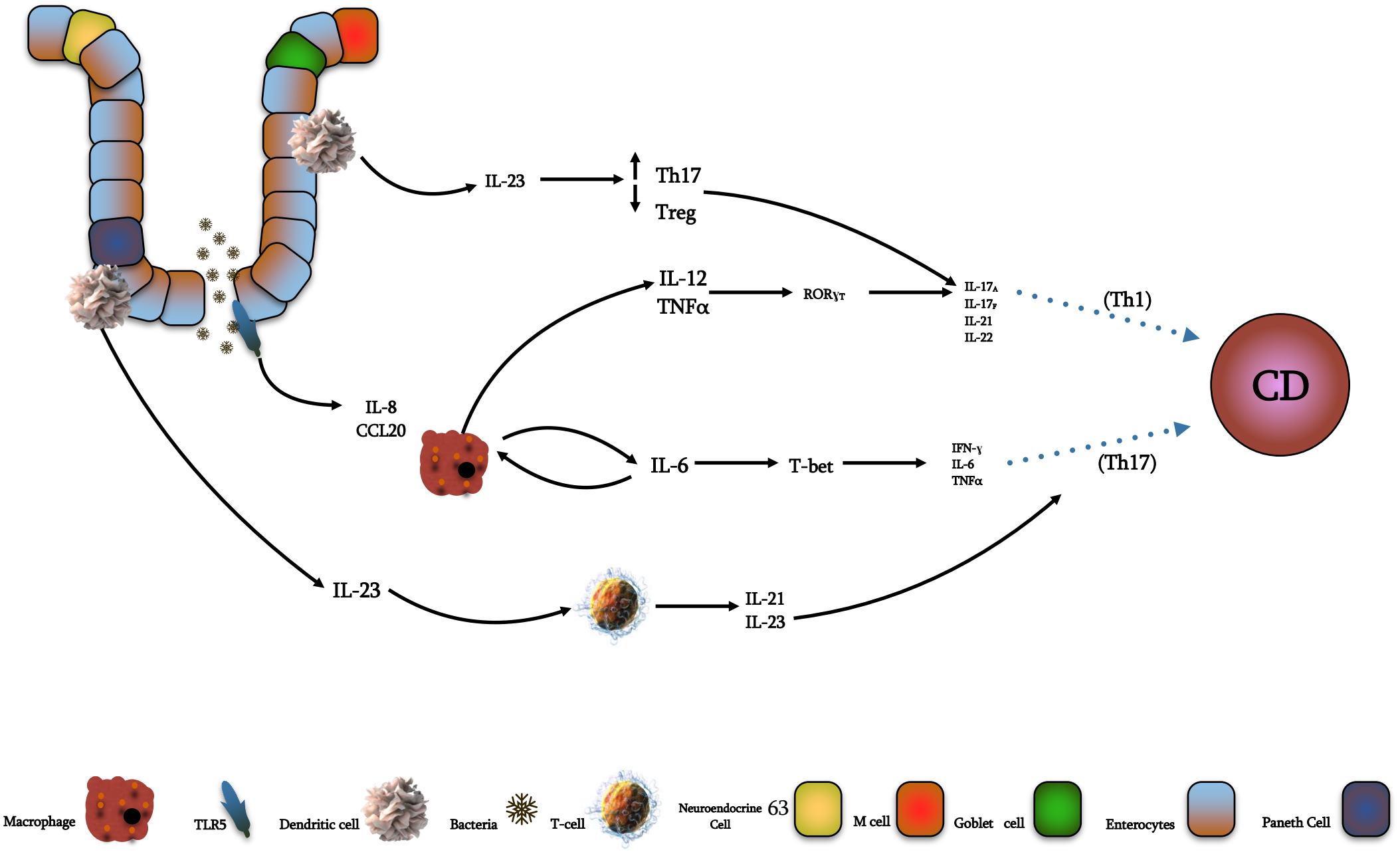
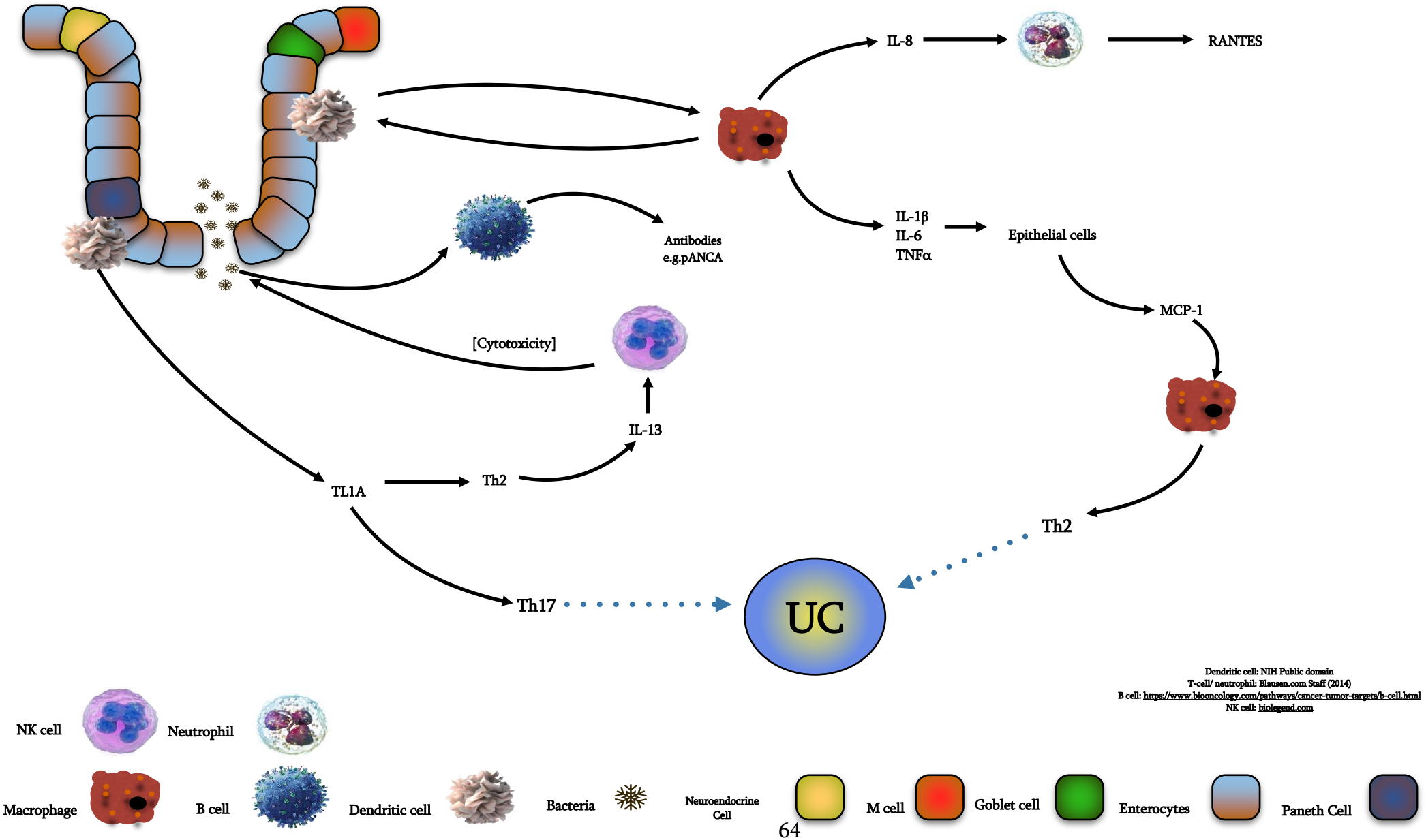


Figure 6. The pathogenesis of ulcerative colitis



Dendritic cell: NIH Public domain
T-cell/ neutrophil: Blausen.com Staff (2014)
B cell: https://www.biooncology.com/pathways/cancer-tumor-targets/b-cell.html
NK cell: biolegend.com

1.17. Cytokines in inflammatory bowel disease

Within inflammatory bowel disease there is a major emphasis on circulating immune cells and their signalling molecules, cytokines. Some, such as pro-inflammatory cytokines, provide a basis for an understanding into the neuro-immune interactions that could be involved in peripheral nociception. Discussed below, IL-1β, IFN-ɣ, IL-6, IL-8, and TNFα, are all considered integral into the pathology of both CD and UC, and could potentially be important in nociception and peripheral sensitisation. Below are cytokines important not only to IBD pathology, but have an impact on nociception and possibly abdominal pain.

Interleukin 1-beta (IL-1β), a 35kDa pro-inflammatory cytokine, was discovered in the early 1970s. It forms part of a large family of interleukin 1 cytokines that are primarily produced by macrophages and dendritic cells but also T-cells, NK cells, endothelial cells and microglia, playing an important role in immune regulation and inflammation (Wewers, 1997; Zhao, 2013; McAlindon, 1998). The involvement of IL-1β in the inflammatory process is complex. It initiates a diverse range of interactions encouraging systematic inflammatory processes by activating neutrophils thereby increasing IL-8 levels, stimulating COX-2 and PGE_2 production in addition to inducing NGF release and further lead to the release of SP, and nitric oxide synthase which causes downstream production of nitric oxide. Long-term exposure PGE_2 and NGF is relevant in visceral pain as both mediators have been shown to activate and sensitise nociceptors leading to the generation of peripheral hypersensitivity through PKA-dependent increases in $Nav_{1.8}$ and $Nav_{1.9}$ currents. (Rush & Waxman, 2004). Together with TNFα, IL-1β activates the bradykinin receptor B2, on sensory neurons and macrophages which leads to further IL-1β and inflammatory hyperalgesia. TNFα and IL-1β are amongst the first cytokines to be released following tissue injury and through specific receptor binding on sensory neurons, induce a cascade of neurogenic inflammation. IL-1β itself has also been shown to elicit sensory nerve firing in rat DRG's, a process thought to involve modulation of both the slow and persistent sodium ion channels $Nav_{1.8}$ and $Nav_{1.9}$, via p38 MAPK signalling pathways (Binshtok, 2008). Afferent recordings have also shown robust afferent activation when IL-1β is added to afferent endings (Hughes, 2013).

Musculoskeletal pain has also been reported to be strongly associated with IL-1β where binding to the IL-1 receptor leads to upregulation of $TRPV_1$, which also leads to thermal pain hypersensitivity (Obreja, 2002).

Although influential in IBD, IL-1β is also associated with severity of pain in fibromyalgia and arthritic pain, where there is a correlation between patient pain scores and serum cytokine levels (Zhang, 2007).

As involvement in maintaining chronic bowel inflammation is also a feature of IL-1β, studies showing mice with depleted ICE (IL-1β-converting enzyme) have reported a protective phenotype from DSS, suggesting that along with the reported importance of IL-1β in the initiation and maintenance of peripheral sensitisation during inflammation, there remains a strong possibility that this cytokine could be a therapeutic target in visceral pain (Siegmund, 2001). As elevated IL-1β levels have consistently been observed in both affected and unaffected regions of the colon in CD and UC, it is important for this study to understand the local levels of this cytokine (Dionne, 1998; Stevens, 1992; Reinecker, 1993; Reimund, 1996).

Interleukin-6 (IL-6) is also implicated in the pathogenesis of both CD and UC where Th_1 or Th_2 cells lead to increases in IL-6 and other cytokines such as IFN-ɣ, TNFα, and IL-13. Therapeutically, IL-6R-specific antibodies reduce T-cell apoptosis and local levels of IFN-ɣ, TNFα, and IL-1β, resulting in a dampening of the inflammation in mouse models of colitis (Atreya, 2000). Produced by $CD4^+$ T-cells and macrophages in the lamina propria, this 27kDa cytokine mediates its pro-inflammatory effects due to receptor binding to gp130 (IL-6R-β) forming an IL-6-sIL-6R complex on the surface of dendritic cells, macrophages, and T-cells, promoting the release of further pro-inflammatory cytokines (Kai, 2005). Increased levels of IL-6 have been reported in many IBD studies which tend to focus on its main pro-inflammatory properties, but interestingly IL-6 also has the potential to exert anti-inflammatory control by reducing IFN-ɣ, TNFα, and IL-1β levels in areas of ongoing inflammation, although this has not yet been related to IBD (Henderson, 2012; Atreya, 2000; Kai, 2005; Xing, 1998; Reimund 1996).

IL-6 has also been shown to be important in musculoskeletal pain and arthritic pain and electrophysiological studies have demonstrated its ability to cause c-fiber activation (Brenn, 2007). Injection of IL-6 into the knee joints of rats caused potentiation of the C-fiber response to both innocuous and noxious mechanical stimuli. Interestingly, the afferent responses were increased by adding the soluble IL-6 receptor alone, suggesting the abundance of IL-6 in an already inflamed joiny is primed for nociceptive signaling and increased mechanical responses. This was supported by the observation that the soluble IL-6 receptor did not alter mechanosensitvity in non-inflamed joints.

Together with TNFα, IL-6 is also understood to induce central sensitization in animal studies. Fibromyalgia patients who were enrolled in a study to identify serum cytokine levels demonstrated elevated IL-6 which correlated with pain scores. Inflammation consistently shows significantly elevated IL-1β, TNFα, and IL-6, amd serum levels of IL-6 correlate to patient pain scores (Koch, 2007). In addition, IL-6 has been shown to act as a pro-nociceptive cytokine. Hughes and colleagues (2013) demonstrated that IL-6 stimulates visceral nociceptors and increased responses to mechanical stimuli. IL-6 has also been reported to activate myenteric neurons and stimulate colonic contractions in rats, through the activation of $Cav_{3.2}$ channels (Buckley, 2014). $Nav_{1.7}$ channels have also been implicated in the effects of IL-6 on sensory nerve hypersensitivity, and additionally IL-6 has also been demonstrated to mediate neuronal activation in spinal cord injury in humans (Yan, 2012; Pedersen, 2015). IL-6 also induces $TRPV_1$ mRNA translation at peripheral nerve endings via PKA and PKC-mediated phosphorylation thereby modulating neuronal excitability and this adaption may underlie enhances pain sensitivity, particularly thermal pain. When IL-6 is applied to $TRPV_1$ -positive neurons together with TNFα, it causes the release of CGRP which further contributes to neuroimmune signalling and creates a cycle of immune cell activation and neuronal hypersensitivity. Therefore, we also studied this cytokine due to its role in nociception.

Interleukin-8 (IL-8) is released early in the immune response to injury or infection. It is released from epithelial cells, $CD14^+$ macrophages, fibroblasts, and T-lymphocytes, and most notably neutrophils. IL-8 is a neutrophil chemoattractant that attracts and activates neutrophils during mucosal inflammation to enhance their migration from blood into tissue (Struyf, 2005). It has been studied in detail for many years and since its expression was first observed in active IBD mucosal biopsies in the mid 1990s, laboratories have studied its production in CD and UC where it has been shown as a reliable marker for ongoing mucosal inflammation correlating with endoscopic and histological severity which has been linked with pain (Mitsuyama 1994; Walczak, 2012; Nielsen, 1997; Cunha, 2008). IL-8 is involved in promoting inflammatory cascades and levels have been shown to correlate with other cytokines such as IL-1β and TNFα, with clinical measurements such as abdominal pain intensity also being linked with IL-8 levels (Mitsuyama 1994).

Tumour necrosis factor (TNFα) activates fibroblasts, induces epithelial cell death, and influences the activation and production of pro-inflammatory cytokines such as IL-6, IL-8, and IL-1β (Pollard, 1994;

Parameswaran & Patial, 2010; Reimund, 1996). Released predominantly from macrophages, dendritic cells, and T-cells, TNFα can lead to sensitisation of nociceptors and the enhanced synthesis of prostaglandins, which can further activate sensory nerves. For example, supernatants generated from UC biopsies cause hyper-excitability in mouse nociceptors through the actions of TNFα (Ibeakanma, 2009). Subcutaneous application of TNFα is capable of lowering the mechanical activation threshold of C-fibers and TNFα application to mouse DRG's leads to A-fiber and C-fiber activation. This mechanical allodynia in C-fibers has been suggested to be mediated through TNF_{R1} activation leading to modulation of $Nav_{1.8}$ and K_V channels through p38 MAP kinase phosphorylation to alter activation kinetics and change neuronal excitability which is a significant contributor to inflammatory pain and neuropathic pain (Ibeakanma, 2009). Neuronal injury results in changes to excitation and coupled with local application of TNFα a rapid onset of allodynia is observed providing evidence for increased sensitivity of DRG's post-injury. Further to this, overnight incubation of DRG's with TNFα caused a decrease in the I_K and I_A currents, effects comparable to those produced by UC supernatants. TNFα has been also been shown to act directly on mouse visceral afferent endings to induce mechanical hyperalgesia in keeping with previous observations of TNFα causing mechanical allodynia in rat DRG's (Hughes, 2013; Homma, 2002). TNFα is often shown working synergistically with other pro-inflammatory cytokines such as IL-1β and IL-6 to drive peripheral pain in arthralgias and myalgias, and to contribute to central sensitisation in animal models (Zhang, 2007; Koch, 2007). TNFα alters excitability in the CNS decreasing glutamate transporter expression and reduced glutamate uptake is understood to increase pain behaviours in rodents through changes in neuronal processing.

Current biological therapies such as Infliximab, Adalimumab, and Etanercept, target TNFα directly and prevent it from binding to TNF-specific receptors, and have been shown to benefit subpopulations of IBD patients by reducing ongoing inflammation and disease symptoms (Nanda, 2013; Shihab, 2016). Pre-clinical studies have also hinted that these therapies may reduce mechanical hypersensitivity in chronic pain states in rats, providing an additional benefit to targeting this cytokine (Chen, 2012).

The IL-17 family of cytokines was discovered in the 1990s by Rouvier and colleagues (1993). $IL\text{-}17_A$ (referred to from here as IL-17) is a35kDa classical Th_{17} cytokine released from epithelial cells, $CD8^+$ T-cells, NK cells, activated monocytes, and neutrophils, and numerous studies have shown increased expression in IBD and

neuropathic pain conditions where it leads to the formation of IL-6 and IL-8, both of which encourage a continued inflammatory response (Biancheri, 2014; Yao, 2015; Pappu, 2010). Interestingly, the pelvic neurons do not seem to be effected by IL-17 mechanisms meaning that inflammatory bowel conditions involving spinal neurons may have IL-17-specific effects (Motrich, 2016). Rheumatoid arthritis mouse models studying inflammatory pain have associated IL-17 with increases in INF-ɣ, TNFα, IL-1β, COX-2, and neutrophil migration, thereby promoting inflammatory processes (Pinto, 2010; Richter, 2013). IL-17 also contributes to other inflammatory pain such as arthritic pain in rat models where it has led to mechanical hypersensitivity in the knee joint (Richter, 2012). When IL-17 was next studied in the rat DRG's the incubation with IL-17 led to PKB and ERK phosphorylation to enhance the DRG excitability.

IL-17 has also been shown to lead to increases in inflammatory proteases such as MMP-1 and MMP-9 at both the protein and mRNA level (Pinto, 2010). As MMP-9 has previously shown involvement in neuropathic pain conditions it remains likely that both mediators may share some properties in visceral pain (Nuttall, 2007; Liou, 2013). Calcium imaging studies have shown little direct activation from IL-17 itself although incubated DRG's led to an increase in $TRPV_4$ expression, a channel responsible in part for noxious mechanical responses (Richter, 2012). In agreement with this data, IL-$17^{-/-}$ mice also have shown a reduced mechanical sensitivity and studies have also shown enhanced C-fiber excitability from exposure to IL-17 via PKB and ERK pathways (Kim & Taylor, 2011; Yao, 2015). Recently, the FDA approved an IL-17 antagonist for psoriasis, which demonstrates the safety and tolerability of IL-17 targeted therapies, which could be useful in adopting IL-17 as a pain target in the future.

1.18. MMP's as potential mediators of pain in inflammatory bowel disease

Matrix metalloproteinases (MMP's) are a family of proteases that degrade the extracellular matrix (ECM) digesting denatured collagens, in particular cartilage. The basic structure of an MMP consists of a pro-domain, a thiol group, a signal peptide, and a zinc-dependent catalytic domain approximately 170 amino acids in length.

The proteolytic actions of MMP's depend on electrostatic attraction between the carbonyl group found on the ECM portion, and the zinc ion.

Due to the destructive nature of these proteases, MMP's are formed as a zymogen, a pro-enzyme which remains inactive until the active site is exposed, usually by cleavage of the pro-domain (Moore, 2011). In order to remain inactive a coordination of cysteine and the zinc-binding motifs in the catalytic domain prevent water molecules essential for catalysis from binding to zinc (Nagase, 2006). Within the MMP family there exist many subtypes grouped by substrate specificity for example collagenases (MMP-1, -8, and -13), gelatinises (MMP-2, and -9), stromelysins (MMP-3, and -10), and membrane-bound (MMP-14, and -17).

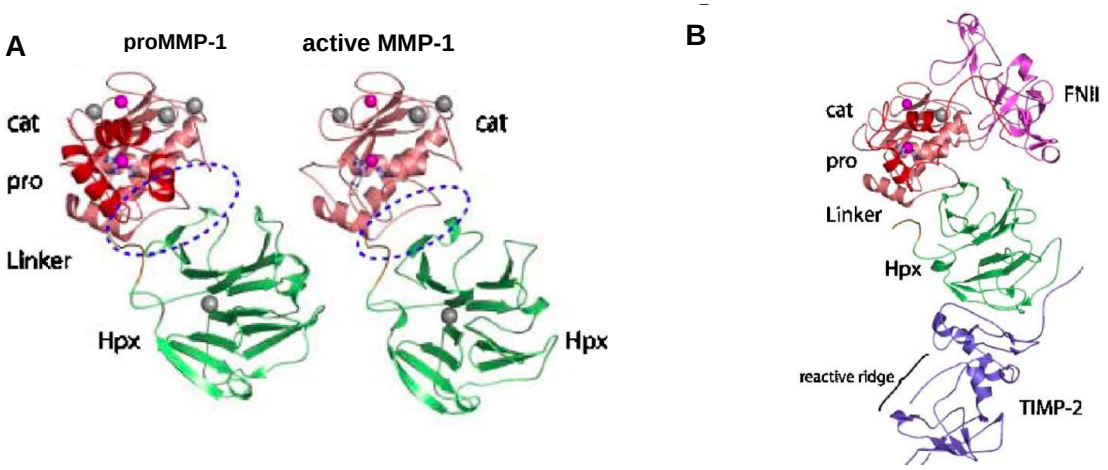


Figure 7. Ribbon diagram of human MMP-1.

(A) Ribbon diagram of human pro-MMP-1 and active MMP-1. **(B)** Ribbon diagram showing pro-MMP-1 and TIMP-1 binding complex. Red indicates the pro-domain, pink indicates the catalytic domain, yellow indicates the linker region, green indicates the hemopexin domain. The purple and grey dots indicate zinc and calcium ions, respectively. Diagram from Nagase *et al.* (2006).

MMP's have been linked to the pathophysiology of IBD, and increased production is observed in CD and UC patients compared with control groups (Moore, 2011; Kofla-Dłuback, 2014; Jimbo, 2014; Matusiewicz, 2014). Techniques enabling specific labelling of areas of the colon have shown strong MMP expression in the mucosal epithelium and lamina propria, in both adult and paediatric IBD patients (Jimbo, 2014; León, 2009; von Lampe, 2000).

Due to the proteolytic nature of MMP's and their abundance at inflammatory sites, they may be involved in the nociceptive pathway. For example, serum levels of MMP-9 have been linked to pancreatic pain although the mechanisms are not yet understood (Wen, 2009). Other proteases such as mast cell tryptase and neutrophil elastase are known to interact directly with sensory afferents through the activation of protease activated receptors (PARs), and the potential for MMP's to act in a similar manner remains to be fully explored.

In addition to being stored as a zymogen, MMP activity is also tightly controlled by the activity of endogenous inhibitors called TIMPs (**t**issue **i**nhibitors of **M**M**P**'s). Four subtypes are known to exist, although so far only the actions of TIMP-1 and TIMP-2 are understood in detail. TIMP-1 and TIMP-2 were discovered for their erythroid potentiating activity and knockout studies suggest that TIMP-2 reduces the cleavage and activation of pro-MMP-2,(Stetler-Stevenson, 1992; Caterina, 2000; Wang, 2009). The TIMP's interact with MMP's by competitive inhibition of the substrate and can also form complexes with MMP's themselves leading to proteolytic destruction (Moore, 2011).

Several MMP's have been strongly linked to IBD and are discussed below as candidates for pain in IBD.

1.18.1. MMP-1

MMP-1, is a gelatinase and cleaves type I, II, and III, collagen in the extracellular matrix. MMP-1 activity increases levels of monocyte chemoattractant protein-1 (MCP-1) promoting macrophage infiltration and initiating further MMP activation cascades. MMP's are also linked to the production of other cytokines, which in turn can increase MMP levels further, for example, IL-6 has been shown to increase MMP-1 levels (Cutler, 2017).

MMP-1 expression is increased in IBD patients, with transcript and protein levels correlating with the severity of inflammation, particularly in UC leading to the suggestion that MMP's could be used as a biomarker for disease progression (Wang, 2009; von Lampe, 2000; Jimbo, 2014). MMP-1 was found to cleave the N-terminal extracellular domain of PAR_1; a distinct site from the thrombin binding region (Blackburn, 2008). Recently a laboratory using A549 cell lines has shown MMP-1 cleavage of PAR_2, an important GPCR in visceral

pain mechanisms and given the interaction with PAR's, MMP-1 could show involvement in the visceral pain process during inflammation (Li & Tai, 2014; Grant, 2007).

1.18.2. MMP-3

MMP-3 is a stromelysin that degrades collagen type II, IV, V, IX, X, XI, proteoglycans, fibronectin, gelatine, and elastin. MMP-3 has also been shown to activate other MMP's such as MMP-9, thereby encouraging inflammatory processes (DeSimone, 1999). Elevated cytokines in IBD can lead to increases in local MMP-3 levels suggesting an interplay between inflammatory mediators in the inflamed bowel (Pedersen, 2015). MMP-3 levels are elevated in IBD patients particularly in UC and a 2008 study by Gordon and colleagues found that a likely source in the inflamed gut comes from IgG-producing plasma cells that secrete large quantities of MMP-3 (von Lampe, 2000; León, 2009; Gordon, 2008).

MMP levels correlate with the severity of inflammation in the colon of IBD patients and inhibition of MMP-3 attenuates colitis in the mouse DDS model (Kolho, 2014; Jimbo, 2014; Kobayashi, 2006). Although there is little published research regarding the role of MMP-3 in pain, the elevated levels of MMP-3 during inflammation and the ability of MMP's to activate other MMP's and cytokines such as IL-1β, suggests that there may be a role for MMP-3 in inflammatory visceral pain (Schönbeck, 1998).

1.18.3. MMP-9

MMP-9 is a gelatinase released by monocytes, epithelial cells, neutrophils, and macrophages (Moore, 2011; Castaneda, 2005). Increased expression of MMP-9 is observed following peripheral and central injury, for example, Liou and colleagues (2013) noted a change in MMP-9 expression in mouse sciatic nerve ligation although the authors failed to mention any measurements of the endogenous TIMP levels which makes the relevance of these results difficult to interpret. In the CNS, microglia have been shown to release MMP-2 and MMP-9 under inflammatory conditions and mouse models of acute spinal cord injury observed increases in MMP-9 levels (Nuttall, 2007). A study by Li *et al.* (2016) attributed MMP-9 (and to a lesser extent, MMP-2) to the progression of neuropathic pain following peripheral nerve injury in rats. It was noted that mechanical and thermal nociceptive responses were significantly reduced when an MMP-2/-9 inhibitor was given

chronically, although interestingly, directly injecting either MMP-2 or MMP-9 resulted in mechanical hypersensitivity only, with no thermal sensitisation, suggesting differential signalling when in isolation but a synergistic effect when both are present, an event which likely mimics endogenous release. The authors suggested that modulation of NMDA-receptors in the spinal cord may be involved, in addition to increased production of IL-1β. Other studies have shown reduced neuropathic pain in MMP-$9^{-/-}$ mice, suggesting different mechanisms underlie the actions of MMP's at central and peripheral sites (Yamamoto, 2003; Kawasaki, 2008). For example, MMP-9 has also been implicated in the production of peripheral mechanical allodynia following CFA injections, and it is likely that this effect is driven by TNFα mediated MMP-9 production (Kular, 2012).

MMP-9 levels are increased in IBD and it is thought to play an important role in the progression of inflammation. In IBD, epithelial-derived MMP-9 attracts neutrophils to the site of tissue injury leading to an inflammatory cascade of cytokines and immune cells. However, increased neutrophil migration has also been observed in MMP-9-deficient mice meaning the role of MMP-9 during inflammation warrants further investigation (Castaneda, 2005). In general, knockout mice show a protective effect from colitis in DSS and TNBS models, with reduced leukocyte recruitment to the intestines (Moore, 2011; Cataneda, 2005). Serum levels of MMP-9 correlate with visceral pain severity and inflammatory scores in pancreatitis and paediatric CD (Matusiewicz, 2014; Kofla-Dłubacz, 2014). Additionally, MMP-9 is is also implicated in the production of TNFα, IL-1β, IFN-ɣ, and SP, mediators important within inflammatory and nociceptive cascades (Shubayev & Myers, 1999; Schönbeck, 1998). In particular, TNFα acts to induce MMP-9 expression within injured peripheral nerves, and MMP-9 has been shown to mediate TNFα-induced recruitment of macrophages (Shubayev, 2006).

1.18.4. MMP-12

MMP-12 was first described in 1981 by Banda and Werb as an inflammatory protein released from macrophages (Banda & Werb, 1981). Since then, it has been shown to be expressed by both the pro-resolution subtype M2 macrophages and the pro-inflammatory M1 macrophages (Marchant, 2014; Shapiro, 1993). M2 macrophages show increased phagocytic activity and higher expression of IL-10 and CD25 and are

associated with Th_2 mediated inflammatory responses during which M2 macrophages are thought to promote resolution of inflammation and tissue remodelling.

Although macrophages are a significant source of MMP-12 they are not the only cell type to produce MMP-12. Within the last decade MMP-12 production has been associated with a variety of cell types including human airway smooth muscle cells, corneal epithelial cells, cultured oligodendrocytes, and neurons affected by hypoxic ischemia in neonatal mice (Lyu & Joo, 2005; Larsen and Yong, 2004; Svedin, 2009). In particular human colonic epithelial cells express MMP-12 along with other MMP's such as MMP-1, MMP-3, MMP-7, MMP-9, and MMP-10 (Pedersen, 2009).

MMP-12 has been implicated in the pathophysiology of several disorders such as asthma, COPD, and IBD, where inflammation and fibrosis are dominant clinical features. In addition, MMP-12 has also been implicated in the pathogenesis of multiple sclerosis due to its role in microglial activation, demyelination, and axonal degradation in multiple sclerosis (Vos, 2003; Stawski, 2014). However not all the actions of MMP-12 are detrimental, for instance, respiratory viral immunity studied in HeLa cell lines and rodents have suggested that immunity was linked to secretion of IFN-α via an MMP-12-mediated uptake within the nuclei of the cells, and MMP-12 has also demonstrated inhibitory properties against bacteria such as *staphylococcus aureus* and *E.Coli*, found in the gut (Marchant, 2014; Houghton, 2009).

Although elevated in IBD its precise actions under inflammatory conditions within the gut are not clear. Other inflammatory conditions such as arthritis have demonstrated that MMP-12 can promote resolution of inflammation by inactivating the C3a and C5a complements, thereby reducing leukocyte attraction (Bellac, 2014). However, other studies looking at acute peripheral inflammation have noted that injecting MMP-12 directly into the mouse ear caused skin inflammation with the rapid recruitment of lymphocytes, neutrophils and macrophages (Nakogomi, 2015). A characteristic of MMP's and in particular MMP-12 is the ability to cleave the pro-domain of other MMP's resulting in activation. For example, MMP-12 can activate MMP-2 and MMP-3 which further activate other MMP's (Ogata, 1992; Shapiro, 1993). This process results in a continued inflammatory environment and as MMP's can be toxic at high concentrations, dysregulation on MMP-12 could pragmatically influence inflammation and possibly contribute to visceral pain (Vos, 2000).

The MMP-12 processes involved in IBD may be relevant in treatment-refractory patients. Approximately 40% of patients fail to respond to anti-TNFα therapy and recently, MMP-12 has been shown to effect the anti-TNFα antibodies infliximab and etanercept (Biancheri, 2015). The authors showed that *in vitro* the combination of MMP-3 and MMP-12 could cleave infliximab and etanercept. The functional effect on infliximab was limited however, but when TNFα was exposed to IBD homogenates a sharp reduction in the TNFα—binding ability was observed, an effect which was reversed with the addition of the multi-MMP inhibitor Marimastat. These studies highlight the importance of MMP's not only in the progression of inflammation but may be responsible for therapy related efficacy.

1.19. Biopsies

A considerable advantage to the field of gastroenterology is the availability of human disease tissue allowing human pathophysiology to be investigated in detail. Generally, tissue samples are obtained by biopsy of the mucosal layer from consenting patients as part of routine clinical investigation. Biopsy samples will normally be taken as a matter of course during these procedures and so the use of biopsy samples to study human disease will remain an established tool for studying GI diseases due to the minimal additional time, cost, and discomfort to patients. By varying incubation time in buffer or culture medium different cellular processes can be investigation. This study utilises biopsy tissue from patients to study changes in the gut environment and a therefore consideration for the biopsy incubation is discussed below.

Acute incubation periods enable the ongoing release of endogenous mediators important to the pathology of GI diseases to be measured. An incubation time of 1-2 hours is sufficient to detect significantly raised mediator levels between diseased (IBD and IBS) and normal tissue. In addition, the introduction of a chemical pH stabiliser, hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) produced more consistent results due to effective preservation of protein structures within the tissue (Baicu & Taylor, 2002).

Within the previous 15 years a more targeted approach to utilising biopsies for the study of chronic abdominal pain has been used. Theses studies have collectively identified altered levels of neuro-immune mediators in tissue from IBD and IBS patients, and characterised their effects on changes in sensory and motor neurons using electrophysiological recording techniques, calcium-imaging, and optical recordings

(Corvera, 1999; Barbara, 2004; Cenac, 2007; Barbara, 2007; Buhner, 2009; 2014; Hughes, 2009; Balestra, 2012; Valdez-Morales, 2013).

Acute incubations afford the opportunity to study mediators of high concentration in the biopsy samples in a logistically simple manner. By contrast chronic incubation of human biopsies affords the opportunity to study cellular events where assay sensitivity could be an issue, for example cytokine release is routinely measured with the duration of incubation ranging from 6-48 hours depending on the specific cytokine family of interest. In addition, chronic incubation of biopsies has also been used to understand the role of infiltrating immune cells such as macrophages and their released mediators (Lloyd, 1975; Schreiber, 1995; Ligumsky, 1997). Chronic incubations allow appreciable levels of inflammatory mediators to build up relative to acute incubation, thereby aiding their detection in quantitative assays (Schrieber, 1995; Reddy, 2007). Chronic incubations also provide the opportunity to manipulate the exogenous environment, for example, Mahida and colleagues used a 24 hour incubation to understand the effects of 5-ASA inhibition of IL-1β production, a cytokine key to promoting inflammation which also shares the capacity to stimulate nociceptors (Mahida, 1991). Similarly, a $CD3^+$ T-cell antibody, otelixizumab, has recently been tested against inflamed mucosal biopsies and LPMC's from IBD patients, where several pro-inflammatory cytokines and chemokines were reduced to control levels, via mechanisms promoting the production of IL-10 (Vossenkämper, 2014). As with all laboratory assays each have their individual strengths and weaknesses. Acute incubations may be limited in the mediators that are detectable, but chronic incubations may also be an additional step away from an accurate depiction of the ongoing disease *in situ*. This current project focuses on acute incubations to provide insight into the mediators responsible for afferent activation in gastrointestinal diseases.

1.20. History of the extracellular recording technique

Electrophysiological recordings of colonic afferents originated from skin nerve experiments in the 1960's. By the late 1980's David Grundy's laboratory identified mechanical, thermal, and chemical properties of vagal afferents using extracellular platinum electrode recording techniques (Collman, 1984; Blackshaw, 1987). Advances in technology later meant that cleaner and more sensitive recordings could be made by introducing biological and scaling amplifiers (Page, 2005). This work was followed up by Brierley and colleagues (2004)

who characterised spinal afferents by adopting platinum electrode recording techniques. Advances in the signal:noise ratio by introducing differential amplification which utilised an electronic amplifier to focus only on the two input voltages (reference and biological) while suppressing any voltage common to both improved the recording technique. Furthermore, improved filtering and computer software for recording and waveform analysis in recent years meant added precision compared with older magnetic tape recordings. The electrophysiological recording technique used in this study is almost identical with the exception of now utilising glass suction electrodes and improved digital and band pass filtering, which can remove electrical noise and allow only signals between set frequencies to be recorded (Hockley, 2014).

1.21. Choosing quantitative polymerase chain reaction

To understand the levels of mediators such as MMP's within the biopsy, qPCR was utilised and chosen above other techniques. Below, are important factors taken into consideration and feature heavily as the cornerstone of transcriptional analysis throughout this study.

1.21.1. Reverse transcription

This research utilised two-step quantitative reverse transcription-PCR (qRT-PCR) which involved RNA extraction from biopsy tissue which was then, in a separate and single tube, converted to cDNA using non-specific forward and reverse primers and a reverse transcriptase RNA-cDNA polymerase. After RNA extraction, the RNA elute is analysed using a nano drop spectrophotometer. This produces 2 ratios of absorbance, (i) the 260/280 nm ratio, which reports on the 'purity' of the RNA and accounts for any protein contamination, and generally an absorbance ratio of 2.0 is ideal for uncontaminated RNA. (ii) The 260/230 nm ratio, reports any chemical contamination in the form of phenol rings, with the usual contaminant being ethanol which is used during the extraction phases to remove cellular material. Here a ratio of 2.0-2.2 are common for any uncontaminated sample. The two ratios work to produce a yield of RNA in ng/µL. In the reverse transcription step, a 100% efficiency is expected where 100ng of RNA would be converted to 1 µg of cDNA.

1.21..2. Taqman probes vs SYBR green

Taqman- MGB (minor groove binding) probes are short, specific sequences of nucleotides that are complementary to regions of cDNA on the gene of interest, and bind between the two primers. The probes contain a reporter dye on the 5' end which is a fluorescent dye to report amplification and a quencher on the 3' end of the probe. This serves a dual purpose, to quench fluorescence of unbound probes and as it sits on the 3' end of the probe, and ensures that it is not extended and amplified due to the DNA polymerase. This study used Taqman probes.

SYBR green dye (synergy brands green dye) will bind to the minor groove of any double stranded DNS (dsDNA). It is not as specific as taqman and as a result, a melting point analysis is required after the 40 cycles

of PCR. When the dye melts (dissociates) at a high temperatures due to the denaturing of the target dsDNA there should be one specific melting point for this. The concept is that there should only be one melting point where specific binding of the dye has occurred, and if the analysis shows more melting points differing significantly from one another, then non-target products will have bound dye attached.

1.21..3. Primer selection

For the purpose of the microfluidic card for performing qPCR, only inventoried primers were selected. However, several considerations were still required for selecting primers that would be suitable for use during the microfluidic card as the PCR cycle temperatures would need to be optimal for all primers for the different genes of interest. The ideal primer should be short enough to be specific, with an ideal number of nucleotides of between 18-28, and not contain lengths of repeated nucleotides which could lead to non-specific binding. For optimal results, it is suggested that there is approximately 50% GC content which helps to prevent any mismatches becoming stable. All primers should have similar melting points, with less than 1°C difference between them. Primers used in this study were inventoried as per the requirements for a custom taqman array card which can be found in appendix E. To understand the melting temperature, the temperature at which half of the primers are annealed to the target region, the following equation can be used:

$$Tm = [(\text{number of G + C}) \times 4^{\circ}C + (\text{number of A + T}) \times 2^{\circ}C)]$$

1.21..4. Reference genes

Three reference genes were used in the Taqman microfluidic card; GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 18s, and β-actin. These genes were picked which have a high expression in mammalian cells and remain stable despite conditions such as IBS and IBD. A total of three genes were used to give the best chance of stable gene expression, and the geometric mean of all three was used in data analysis (Vandesompele, 2002). The geometric mean calculation was used as shown:

$\sqrt[3]{\text{GAPDH value + 18s value + beta-actin value}}$

The geometric mean provides a more reliable mean value based on the relationship between each number, so that normal fluctuations in expression that are to be expected under normal circumstances all provide the same input into the mean value.

1.21.5. Contamination

Preventing contamination of genomic (g) DNA using uracil-n-gylcosylate (UNG) is an effective step in reducing false positives. The UNG will degrade any contaminating uracil-containing PCR product, and leave the natural thymine-containing target DNA template unchanged. At the start of the PCR reaction, a 10 minute incubation at 50℃ is enough for the UNG to work, which is then inactivated as the PCR reaction heats up to 95℃ for the first cycle.

1.21.6. Threshold cycle

The threshold was automatically set at 10 times the standard deviation of the fluorescence value at baseline (the signal during the initial 3-15 cycles). The threshold cycle (C_T) is an important value in qPCR and is used for analysing the relative abundance of material and any fold-change to control tissue also collected. The C_T is set at the cycle number at which the fluorescence signal of the reaction surpasses the threshold. This will be different for each gene of interest depending on the expression level. When compared to housekeeping genes (reference genes) which are abundantly expressed, a relative expression of the gene of interest can be compared. Generally, the lower the C_T value is, the more cDNA and in turn RNA there is.

1.22. GENERAL METHODOLOGY

1.22.1. Patient consent and pain scoring

Ethics approval for the study was provided by the East London and The City Health Authority Research Ethics Committee (REC# P/01/023). Patients were consented on the day of biopsy collection before the patient underwent endoscopy. Patients were asked for their highest level of pain in the previous 4 weeks prior to biopsy, and on the day of biopsy collection, using an ascending 0-3 scale (0: no pain; 1: mild pain; 2: moderate pain; 3: severe pain).

1.22.2. Biopsy collection and supernatant generation

A total of 4 biopsies were taken per patient from the colon (IBD and FAP patients) or the terminal ileum (Crohn's disease). All patients were under 18 years of age (5-17 years). IBD samples were taken from sites of inflammation. Control biopsy samples were taken from endoscopically normal tissue from patients undergoing endoscopy for polyp surveillance who showed no clinical signs of intestinal inflammation or disease and reported no abdominal pain. Biopsy samples were placed in an eppendorf tube filled with Krebs/Hepes buffer (In mM: 1.7 MgCl; 1.7 CaCl; 1.2 NaH_2PO_4; 135 NaCl; 3 Hepes; 12.2 glucose; 5.4 KCl; Sigma-Aldrich, UK), 2 biopsies per tube and transported on ice to the laboratory (transport time 20min). Biopsies were then weighed and placed in 0.5 ml fresh Krebs/Hepes buffer and oxygenated at 37°C for 60 minutes. Samples were removed and centrifuged for 20 minutes at 12500 x g, using spin-x tubes (Sigma, UK). The supernatant was removed and aliquoted to be stored at -80°C until needed. Biopsies were re-weighed and stored at -80°C in a covering volume of RNA*later* (Invitrogen, UK) until later use in qPCR studies. Researchers were blinded to patient details and phenotype until after analysis of the data from these studies.

Patient	Phenotype	Age	Gender	Initial clinical presentation	Pain Score 24Hr	Pain Score Month	Macroscopy	Histology	ESR	CRP	Inflamed	Medication
1	**FAPS**		F	ABDO PAIN/ HYPERMOBILITY	2	2	NORMAL	NORMAL	10	<5	NO	MEBEVRIN
2	**FAPS**	14	F	ABDO PAIN/ BLEEDING	1	3	NORMAL	NORMAL	12	<5	NO	NONE
3	**FAPS**	8	M	ABDO PAIN	0	1	NORMAL	NORMAL	<5	<5	NO	NONE
4	**FAPS**	12	M	ENTEROPATHY	1	2	NORMAL	NORMAL	2	<5	NO	PREDNISOLONE/ PARACETAMOL
5	**FAPS**	11	M	ABDO PAIN/ HYPERMOBILITY	2	3	SPASMODIC	NORMAL	18	<5	NO	NONE
6	**FAPS**	3	F	BLEEDING	0	1	NORMAL	NORMAL	<5	<5	NO	NONE
7	**FAPS**	12	F	ABDO PAIN	0	1	NORMAL	NORMAL	<5	<5	NO	PARACETAMOL
8	**FAPS**	10	F	ABDO PAIN	0	2	NORMAL	NORMAL	<5	<5	NO	BUSCUPAN
9	**FAPS**	15	F	ABDO PAIN	0	1	NORMAL	NORMAL	3	<5	NO	NONE
11	FAPS	12	F	ABDO PAIN	1	3	NORMAL	NORMAL	<5	<5	NO	PARACETAMOL
12	**CD**	10	M	NEW DIAGNOSIS	0	1	CD	CD	<5	10	YES	NONE
13	**CD**	12	M	NEW DIAGNOSIS	1	1	CD	CD	104	<5	YES	MEBEVRIN
14	**CD**	16	F	NEW DIAGNOSIS	2	2	CD	CD	80	<5	YES	NONE
15	**CD**	15	M	CD	0	1	COLITIS/ILEITIS	COLITIS/ILEITIS	16	<5	YES	AZATHIOPRINE/ INFLIXIMAB
16	**CD**	5	F	NEW DIAGNOSIS	0	1	NORMAL	CD	8	<5	YES	NONE
17	**CD**	12	M	NEW DIAGNOSIS	1	2	PATCHY INFLAMED	CD	65	25	YES	NONE

18	**CD**	10	M	NEW DIAGNOSIS	0	1	CD	CD	45	40	YES	NONE
19	**CD**	13	F	CD	2	2	CD	CD	42	18	YES	AZATHIOPRINE/ INFLIXIMAB
20	**CD**	16	F	CD	1	1	NORMAL	CD	12	19	YES	AZATHIOPRINE/ PENTASA
21	**CD**	17	M	CD	0	1	CD	CD	29	<5	YES	AZATHIOPRINE/PREDNISOLONE
22	**CD**	16	M	CD	1	1	CD	CD	<5	<5	YES	INFLIXIMAB/AZATHIOPRINE
23	**CD**	7	F	NEW DIAGNOSIS	0	1	COLITIS	CD	<5	<5	YES	NONE

Table 4. FAPS and Crohn's disease patient details

Patient	Phenotype	Age	Gender	Initial clinical presentation	Pain Score 24Hr	Pain Score Month	Macroscopy	Histology	ESR	CRP	Inflamed	Medication
24	**UC**	15	M	UC	3	0	ACTIVE COLITIS	ACTIVE COLITIS	<5	14	YES	STEROIDS
25	**UC**	13	M	NEW DIAGNOSIS	3	2	COLITIS	COLITIS	15	22	YES	PARACETAMOL
26	**UC**	8	F	NEW DIAGNOSIS	0	1	PAN COLITIS	COLITIS	20	<5	YES	NONE
27	**UC**	13	M	NEW DIAGNOSIS	2	2	PAN COLITIS	COLITIS	44	<5	YES	NONE
28	**UC**	15	M	ABDO PAIN	0	1	ULCERS	COLITIS	<5	<5	YES	NONE
29	**UC**	16	M	UC	0	2	PAN COLITIS	COLITIS	<5	18	YES	MESALAZINE/ AZATHIOPRINE
30	**UC**	15	M	ABDO PAIN	1	1	COLITIS	COLITIS	5	<5	YES	NONE
31	**UC**	16	F	UC	2	2	COLITIS	COLITIS	<5	13	YES	INFLIXIMAB/METHOTREXATE
32	**UC**	2	M	BLEEDING	0	1	COLITIS	COLITIS	<5	<5	YES	METRONIDAZOLE/CIPROFLOXACIN
33	**UC**	15	F	BLEEDING	1	1	COLITIS	COLITIS	30	<5	YES	NONE
34	**CONTROL**	2	F	CONSTIPATION	0	0	NORMAL	NORMAL	<5	<5	NO	NONE
35	**CONTROL**	12	M	DIARRHOEA	0	0	NORMAL	NORMAL	10	<5	NO	NONE
36	**CONTROL**	6	M	POLYPS	0	0	NORMAL	NORMAL	<5	<5	NO	NONE
37	**CONTROL**	6	M	BLEEDING	0	0	NORMAL	NORMAL	25	9	NO	NONE
38	**CONTROL**	9	F	POLYPS	0	0	NORMAL	NORMAL	<5	<5	NO	NONE

Tasble 5. Ulcerative colitis and control patient details

1.22.3. Electrophysiological recordings of colonic splanchnic afferent

Male C57BL/6 mice (12 weeks) were euthanised by rising concentration of CO_2 and cervical dislocation in accordance with Schedule 1 of the Home Office Animal Scientific Procedures Act (1986). The distal colon with associated lumbar splanchnic nerves was removed and the colon opened along the anti-mesenteric border and pinned flat mucosal side up. The tissue was perfused (7ml/min; 32-34℃) with carbogenated Krebs buffer (In mM: 124 NaCl; 4.8 KCl; 1.3 NaH_2PO_4; 2.4 $CaCl_2$; 1.2 $MgSO_4.7H_2O$; 11.1 glucose; 25 $NaHCO_3$) and supplemented with 10µM nifedipine and 10µM atropine to block smooth muscle contraction, and 3µM indomethacin to inhibit endogenous prostanoid production.

Single unit activity was discriminated using wave form analysis software (Spike 2 Cambridge Electronic Design) from recordings of activity in fibers teased from the lumbar splanchnic nerve (rostral to the inferior mesenteric ganglia), using borosilicate glass suction electrodes. Spike 2 software was used discriminate between multiple nerve fibers based on the shape and amplitude of indivual action potentials during post-hoc wavemark analysis to ensure that each individual nerve fiber was assessed independently of other fiber activity. In some instances, multiple fibers were recorded but only fibers with receptive fields within the application ring were assessed and used within the data. Three receptive fields per colon were used and each experiment utilized a new receptive field for each incubation.

Signals were amplified, band pass filtered (gain 5K; 100-1300 Hz; Neurology, Digitiser Ltd, UK), digitally filtered for 50 Hz noise (Humbug, Quest Scientific, Canada), digitised at 20 kHz (micro1401; Cambridge Electronic Design, UK) and displayed on a computer using Spike 2 software. Mechanically-sensitive afferents were found by systematically probing the tissue with a soft brush and individual receptive fields of afferent nerve fibers were identified by systematically probing the tissue with a 1g Von Frey hair (VFH). Once identified, units were further characterised based on the protocol devised by Brierley and colleagues (2004). This consisted of examining the response of the

identified unit to circumferential tissue stretch using a cantilever system with a claw attached to the tissue adjacent to the receptive field (5g weight suspended from the cantilever for 30s) and stroking the mucosal layer over the receptive field with a 0.16 g VFh applied 10 times. Units that only responded to focal compression of the receptive field with VFh and not to stretch or stroking were classified as serosal afferents based on the presumptive location of their receptive field in the bowel. Studies were only performed on serosal units due to their role as nociceptors. Once a serosal unit was identified the receptive field was probed with a 1g VFh (duration of probing 3 seconds, 4 probes performed, with the 3 highest used for analysis) (Brierley, 2004; Brierley, 2008) and a metal ring was placed over the receptive field. Ongoing activity was observed for 4 minutes with the last 3 minutes being used to calculate the mean baseline firing. The Krebs solution within the ring was then carefully removed and filled with biopsy supernatant, warmed to bath temperature, from either control, IBS, or IBD patients. Following a 7 minute incubation period the supernatant was removed, the ring taken off the tissue and the receptive field re-probed with 1g VFh within 2 minutes. Preliminary studies showed that the application of vehicle (Krebs:HEPES) to the ring for the 7 min test period (11 min in total) elicited no significant change in afferent activity compared with baseline

1.22.4. Drugs

Stock concentrations were made of nifedipine (10mM; DMSO), atropine (10mM; EtOH), indomethacin (3mM; DMSO), PAR4-AP (10mM; water), TRAP-6 (10mM DMSO).

1.22.5. Electrophysiology timeline

The initial stage of the electrophysiology protocol involves sacrifice of a C57BL/6 mouse as described above. The distal region of the colon is removed and the internal organs are frequently washed with krebs (4°C). The colon is then transferred to the recording chamber where the splanchnic nerve is teased apart and recording of single fiber activity starts. Characterisation of single-units are performed and serosal layer

afferents are selected for the supernatant application. Lastly, the recordings are then analysed.

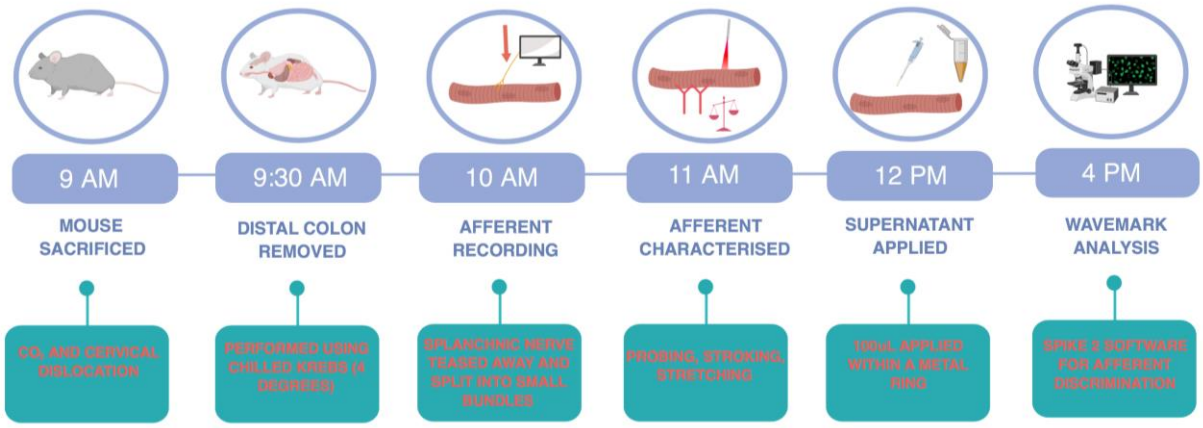


Figure 8. Timeline of electrophysiology experiment. The dissection, recording and analysis would be done on the same day, and researcher was blind to patient supernatant during experimentation.

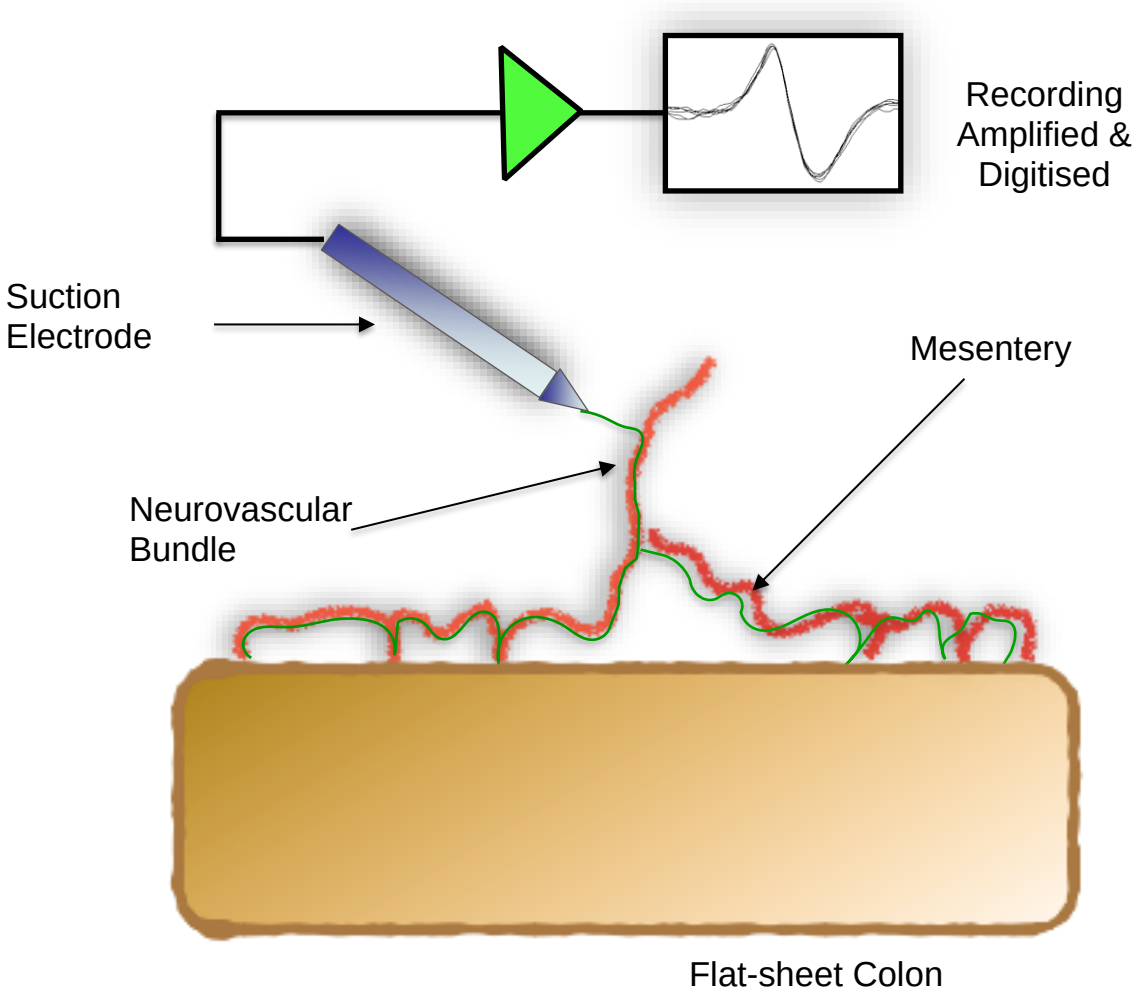
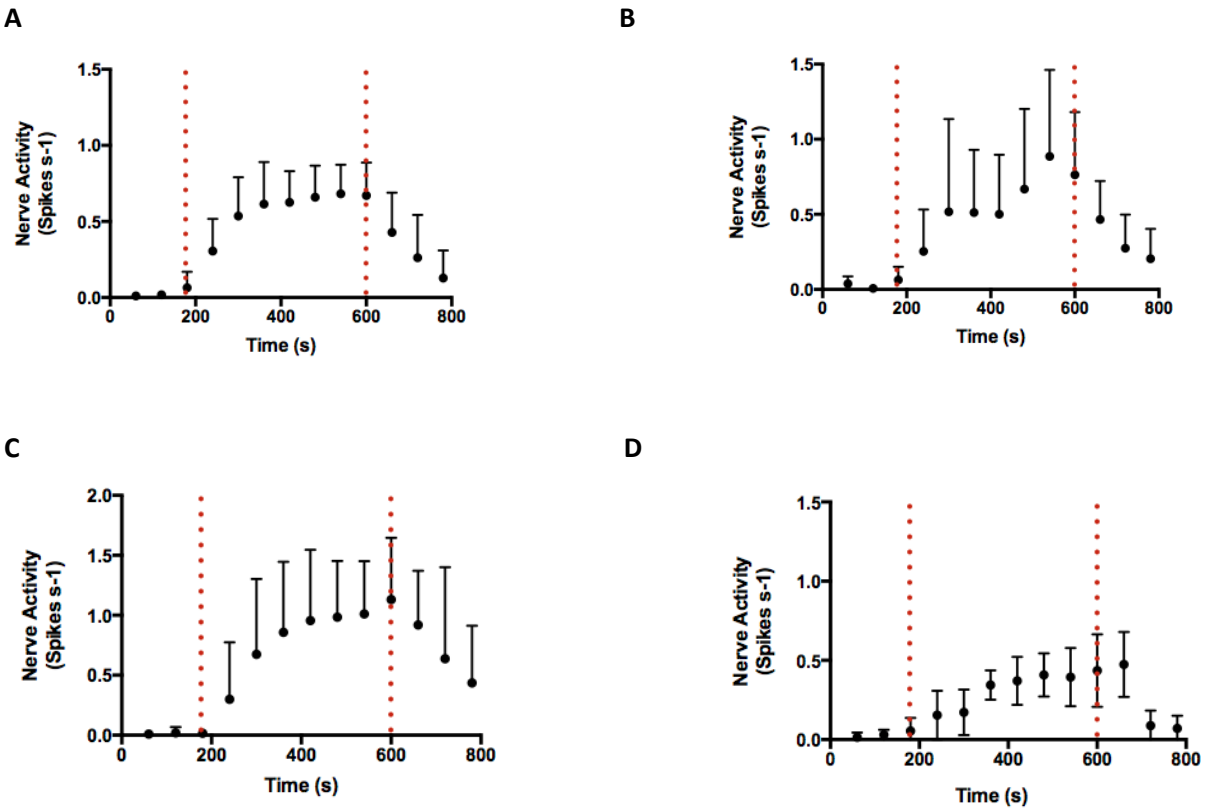


Figure 9. Schematic of nerve recordings on C57BL/6 mice splanchnic nerve.

The colon was cut open at the mesenteric border and pinned as a flat sheet mucosal side up. Receptive fields were found by blunt probing of the tissue and supernatants were applied using a small metal ring over the receptive field.

Example time profiles of the mean response from supernatants on mouse serosal afferents are shown below (figure 10). Followed by an entire recording of an electrophysiology protocol.



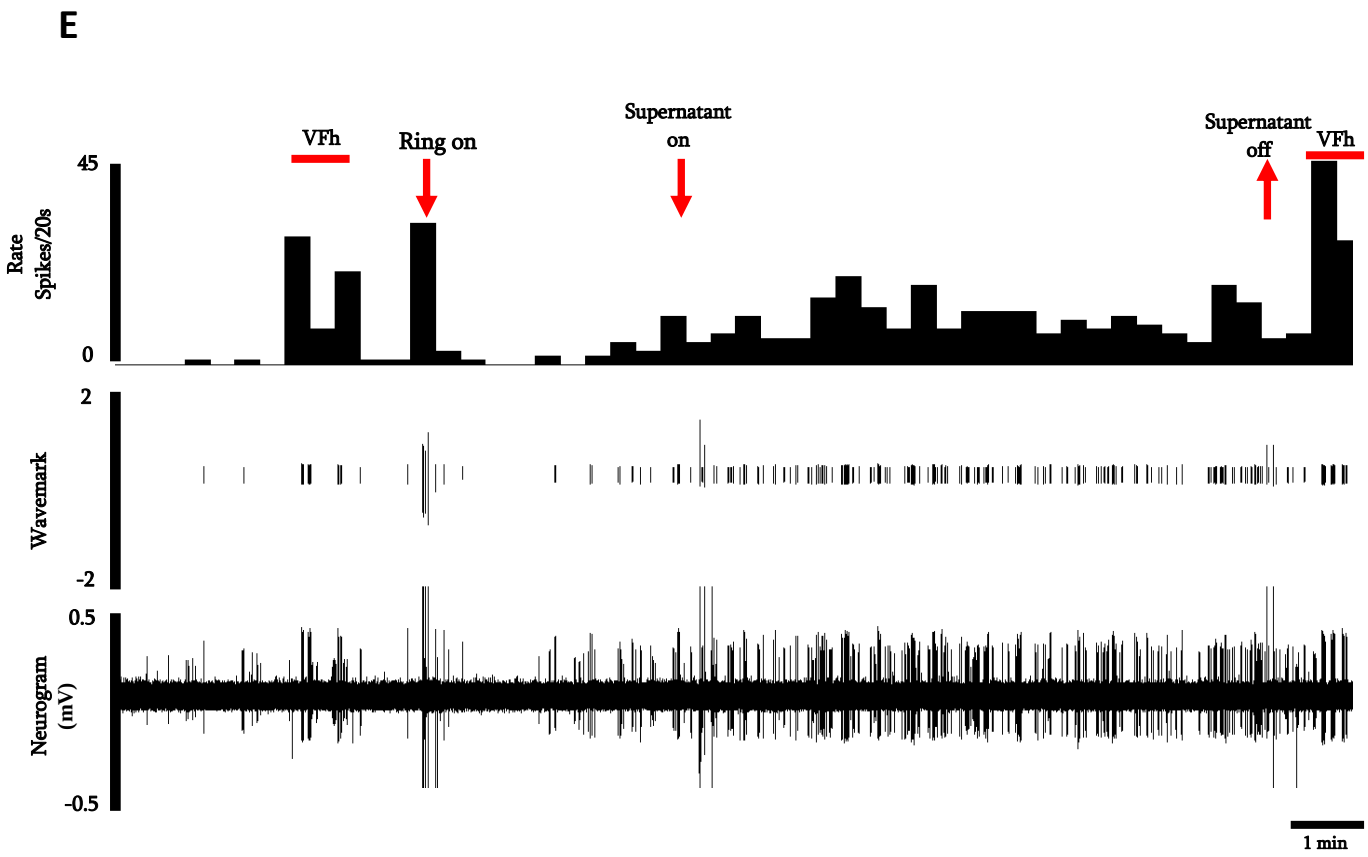


Figure 10. Example trace of electrophysiology

(A) Shows the mean response profile of CD supernatants, **(B)** UC supernatants, **(C)** FAPS supernatants, and **(D)** control supernatants. Between the red dotted lines shows the area of supernatant incubation with each time profile demonstrating afferent activation over 7 minutes. **(E)** Shows an example of a whole electrophysiology experiment using CD supernatant, with the initial VFh probing to test pre-incubation mechanical sensitivity, where the metal ring is added over the receptive field to house the supernatant, also where the supernatant is applied and removed, followed by the ring being taken off (immediately following the removal of supernatant), and final VFh probing. The trace demonstrates afferent activation measured by the lower trace, and wavemarked for accuate analysis as shown in the middle trace, and the raw data plotted as rate histogram on the top trace.

1.22.6. Magpix Luminex analysis of cytokines

Supernatants were analysed for 4 cytokines (IL-1β, IL-6, IL-8, TNFα) using a Magpix Luminex (Luminexcorp, USA) and the multiplex based immunoassay kit (Invitrogen, UK) as per the manufacturers protocol. Antibody-coated beads were sonicated for 30s and 25µL of each bead was added to wells in a 96-well plate and washed in 200µL wash buffer. Next, 50µL of supernatant was added to designated wells and diluted 1:1 in assay diluent and all samples were analysed in duplicate, with the mean concentration used in the results. Serial dilutions of manufacturers standards were used to create a concentration curve. Beads were kept in the dark to prevent exposure to light and kept at room temperature. Samples were placed on an orbital plate shaker for 2 hours at 600rpm and then washed twice in 200µL wash buffer. Next, 100µL of biotinylated antibody was added to each well and shaken at 600rpm for 1 hour, before washing twice in 200µL wash buffer. Next, 100µL of SAV-RPE (Streptavidin-conjugated protein, R-Phycoerythsin) was added to each well before the incubating on a plate shaker at 600rpm for 30 minutes and washing twice in 200µL wash buffer. Finally, 125uL of wash buffer was added to each well and cytokine levels were measured.

1.22.7. RNA extraction from biopsies

The RNA extraction from whole biopsy tissue performed as per the protocol determined by the manufacturer (Qiagen, RNEasy Micro Kit). Briefly, 2-4 biopsies per patient were placed into 350µL RLT buffer and homogenised using a tissue disrupter for approximately 15-20s before centrifuging at 12500 x g, for 3 minutes. Fresh tissue disrupter tips were used for each patient to prevent contamination. The supernatant was removed and 350µL of 70% ethanol was added and mixed by pipetting. Next, the sample was transferred to an RNeasy MinElute spin column and centrifuged for 15s at 12500 x g. Next, 350µL RW1 buffer was added to the spin column and centrifuged for 15s at 12500 x g. Then, 10µL DNase I stock was added to 70µL buffer RDD for each biopsy/patient and gently mixed. This mixture was then added to the spin column and placed at room temperature for 15 minutes. After the 15 minutes, 350µL buffer RW1 was added to the spin column and centrifuged for 15s at 12500 x g. Then, 500µL buffer RPE-ethanol was added to the spin column after a new collection tube was attached, and the sample was centrifuged for 15s at 12500 x g. Next, 500uL 80% ethanol was added to the spin column and centrifuged for 15s at 12500 x g. The spin column was then placed

in a new 2 ml collection tube and centrifuged with the spin column lid open for 5 minutes at 12500 x g to dry the membrane and the collection tube was discarded and replaced with a 1.5 ml collection tube. Next, 15µL RNase-free water was applied directly to the centre of the spin column membrane and centrifuged for 1 minute at 12500 x g and the elute was collected and analysed using nano drop for RNA quality and quantity before being stored at -80℃ or used immediately for reverse transcription.

1.22.8. Reverse transcription

Reverse transcription was performed in guidance with the manufacturers protocol (Applied Bioscience). For each sample, 10µL buffer mixture and 1µL 20x enzyme mixture containing the RNA-DNA polymerase was added to each sample. The volume of sample added was determined by the RNA quality and quantity as measured by Nanodrop. Approximately 1.5-2.0 mg of RNA was added to the reaction tube and the volume was made to 20µL. A negative control was also added with similar quantities of all compounds except the 20x enzyme mixture, and the missing volume was replaced by RNase-free water. The control reaction was used to assess genomic DNA (gDNA) carry-over from the RNA extraction process.

1.22.9. QPCR polymerase chain reaction using Taqman microfluidic array cards

Once the cDNA was shown to express GAPDH within expected values it was then added to the Taqman microfluidic card for multiple gene qPCR. The card used an FAM reporter with an NFQ-NGB quencher on each primer probes, and utilised a ROX passive reference dye. The card was removed from storage at 4℃ and left to reach room temperature. Next, 200ng cDNA was added at a volume of 20µL, with 50µL 2x Taqman universal PCR mastermix, and 30µL RNase-free water, to the loading well of the card. The card was then centrifuged at 12000rpm for 3 minutes at 1 minute intervals to ensure the sample and mastermix filled each of the 48 wells in the card, before sealing it and removing the loading attachment with scissors. The card ran for 40 cycles at 60℃ and 95℃ with each sample gene being measured in duplicate. Three reference genes (housekeeping genes) GAPDH beta-actin were included in the card for each sample and some also contained 18s.

1.22.10. QPCR using GAPDH to assess cDNA quality

Quantitative polymerase chain reaction was performed using Taqman qPCR master mix (Life technologies, UK). Reactions were run in triplicate on a ABI 75000 real-time PCR machine using a 96-well plate. Taqman probes for GAPDH using an FAM reporter and NFQ-MGB quencher were used to detect dsDNA synthesis. Each reaction was performed in 20µL containing 2µL cDNA diluted 1:2, 10µL 2 x Taqman universal PCR mastermix, 7µL RNase-free water, and 1µL primers (forward and reverse). The PCR cycles included 40 cycles between 65℃ and 95℃ proceeded with 10 minutes at 50℃ to activate the UNG mastermix. A reverse transcription negative control was added to ensure the absence of gDNA contamination and a negative control for the dsDNA formation, with no template, was added to observe any significant dimer formation. A mean C_T value for each sample triplicate was obtained and measured against other samples to understand the stability and expression in the original cDNA.

The reference gene GAPDH was used to compare the variability between Taqman microfluidic PCR cards. The mean value for each card was plotted and a 1-way ANOVA with Tukey multiple comparisons test revealed no statistically significant difference between the means. Therefore each card can be grouped together in a total data set with the understanding that each card PCR has been treated similarly. When the Bartlett's test was done, to assess the differences in the variance between the cards, there was a significant difference ($p<0.0001$). Anomalies were found by using 1 C_T value above or below the total GAPDH mean for every sample and they were removed from analysis.

In addition to this, to understand the consistency of each reference gene in the samples, they were grouped together and compared with other reference genes. The geomean was also added to the comparison as this was the value used in the analysis.

Analysis of data used the Livak method, more commonly known as the $2^{-\Delta\Delta Ct}$ method to measure fold change differences or the $2^{-\Delta Ct}$ method (Livak, 2001). This method involves three steps, briefly

I. ΔCt = Ct target - Ct reference

II. $\Delta\Delta Ct = \Delta Ct$ test - ΔCt calibrator

III. $2^{-\Delta\Delta Ct}$

Patient cDNA was excluded from analysis based on assessment of the housekeeping genes. If the geometric mean of a patient's housekeeping genes were greater than 1 C_T value above or below the mean value for all patient's housekeeping genes, the data was not included in the analysis. When transcript levels did not reach above C_T 35 then they were assumed to be below the limit of detection and were excluded from analysis and figures, and were not reported as zero.

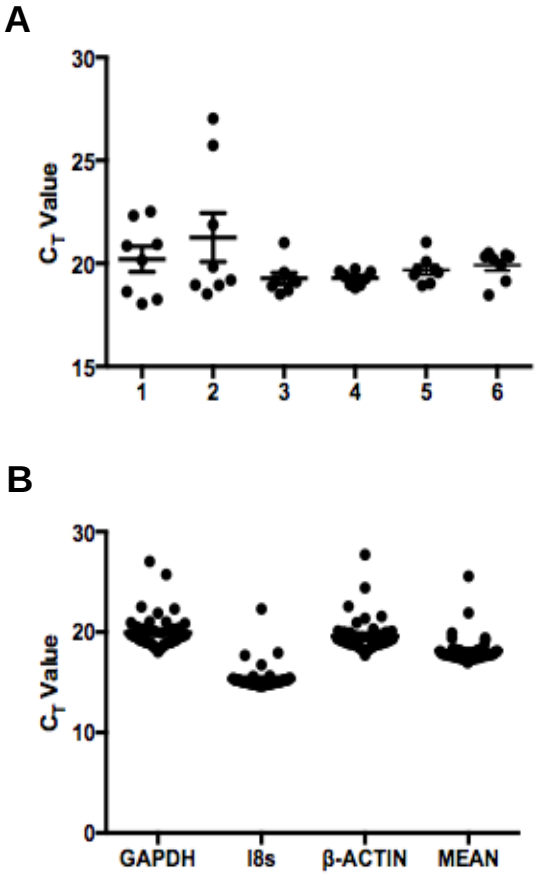


Figure 11. Quality control in qPCR.

(A). The cycle threshold of GAPDH in each Taqman microfluidic card. A total of 6 cards were used to assess genes of interest and to confirm that variability between cards would not affect the overall analysis the GAPDH of each biopsy in all cards were assessed (N=7; ANOVA). **(B)**. The cycle threshold of each of the reference genes. The variability of each of the reference genes for all biopsy samples were compared with eachother to understand if anomalies existed. The mean data set shows the geometric mean on all three reference genes. Lines represent mean ± S.E.M. (N=7 , *n*= 38 ; ANOVA).

1.22.11. Statistical analysis

For data sets within electrophysiology experiments, the Shapiro-Wilk test for normality was performed. A significant p value ($p<0.01$) was observed and therefore the null hypothesis is rejected confirming that the supernatant from patients in this study elicit resonses that are not normally distributed. This data was then subjected to the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison tests. This means that the distribution between data sets is not statistically significant and non-parametric tests can be performed for each patient phenotype. Statistical significance was set at $p<0.05$ and analysed using a Mann-Whitney test for non-parametric analysis.

Figures throughout the study utilise median values with interquartile ranges represented by a flat line with error bars in figures. This is written as a value (interquartile rage). Where the values in a data set were reduced, for example in drug-treated groups, mean values ± standard error mean were used, which gave a more representative value within smaller data sets where the median values did not give sufficient detail, where a students t-test was used for statistical analysis.

The Shaprio-Wilk test was also performed for patient data within the transcriptional analysis using qPCR and showed that the expression profiles do not follow a Gaussian distribution. However, due to the large data set within the expression profiles, a signficance value of $p<0.0001$ was set, to ensure that errors are not incorporated into the data sets through a higher number of test. The Kruskal-Wallis test was also performed. Post-Hoc analysis using Dunn's multiple comparisons showed that there was no statistical difference between the control vs FAPS groups, or between the CD vs UC groups, but significnt differences between all others. Control samples for qPCR contained FAPS data as they were shown to have no significant differences to original control biopsy expression, which enabled statistical testing in IBD expression data.

Therefore, the threshold was set at the mean firing for controls, coupled with studying the raw traces and time profiles of the nerve activity.

Correlations were analysed using linear regression (r^2) and ANOVA was performed on the correlation (p value). This statistical data appears in the results section text before a figure is introduced.

Outliers were identified using the Extreme Studentised Deviate method and the iterative Grubbs' analysis on PRISM software.

1.23. GENERAL RESULTS

Chronic abdominal pain in children has been reported to be as prevalent as 1 in 4, where this pain is often reoccurring for months or years where it has a significant impact on school and home life, sleep, appetite, and overall quality of life (Huertas-Ceballos, 2009; Warschburger, 2014; Chiou, 2010). Chronic abdominal pain often presents as a symptom of IBS, FAPS, or IBD, and a study by Schirbel and colleagues (2010) reported that 40% of adult IBD patients suffered with severe pain during flare-ups. This study was therefore based on understanding the mechanisms of pain and nociception from paediatric patient populations of FAPS, CD and UC. Although abdominal pain is an important clinical feature of these conditions, little is understood about the precise mechanisms of nociceptor activation and incorporating electrophysiological techniques enables modelling of potential endogenous pain mechanisms *in vitro*. Several studies have utilised these recording techniques to understand enteric neuronal activation within the gut but have largely focused on adult IBS patient populations, or specific immune cell mediators that exhibit painful properties (Reed, 2003; Buhner, 2009; Bunher, 2014; Hughes, 2013; Balemans, 2017). It is currently unknown how supernatants generated from patient biopsies will act on colonic serosal afferents and the mechanisms of nociceptor activation in IBD is not understood, hence, this study will observe the effects of supernatants on colonic afferents.

Along with direct afferent activation, visceral hypersensitivity is a key feature of chronic abdominal pain, in particular adult IBS. Barostat studies in patients with IBS, and CRD of mice with inflamed colons have revealed a hypersensitive colon to mechanical stimuli (Barbara, 2007; Crouzet, 2013; Buhner, 2014; Balemans, 2017). Supernatants from this study will also be assessed for their ability to elicit changes to nociceptor threshold potentials for noxious mechanical stimuli.

Although the patients in this study have undergone clinical examinations to stratify them based on diagnosis, the implementation of protein assays for inflammatory cytokines mean that there can be confidence that any stimulation of the afferents from control or FAPS patients is not due to inflammatory processes. IBD is understood to elicit strong cytokine responses in the inflamed gut and several of thes, such as IL-1b, IL-6, and TNFa, have been linked to afferent activation and pain and so they are assessed in this study as a potential mediator for nociception in abdominal pain (Reimund, 1996; Dionne, 1998; Rush & Waxman, 2004; Hughes, 2013; Kai, 2005; Henderson, 2012; Xing, 1998).

The first part of this current study is to understand the feasibility of generating supernatants in order to study colonic afferent responses. The first aim therefore is collect biopsy samples and understand if a supernatant generated for 1 hour will be sufficient to collect pro-nociceptive mediators that can penetrate through to the serosal layer colonic afferent in a concentration great enough to elicit action potentials. Based on previous studies using short incubations of biopsies from adult IBS patients on enteric neurons from mouse colon, it is hypothesised that afferent stimulation will occur. However, the effects of supernatants from paediatric FAPS patients, and paediatric IBD patients is currently unknown and will be studied here. Secondly, as pro-inflammatory mediators such as IL-1β have been shown to directly colonic afferents or mouse DRG's, the second aim of this study is to identify further mediators which may lead to nociceptor activation. As mast cell infiltration and proximity to nociceptors in adult IBS is hypothesised to underlie the initial mechanisms of afferent stimulation which leads to visceral hypersensitivity, this study aims to understand if mast cell mediators may play a similar role in FAPS. Mediators such as 5-HT, histamine, and ATP, have all been shown to robustly stimulate nociceptors although this has not been quantified within a model of nociception which this study will attempt to provide. The last aim of this chapter is to provide evidence of any activation which is observed in a manner that could be beneficial for future studies and therapeutic development, which is the overall target for this study.

1.23.1. AIMS

The aims of this project are as follows:

- To establish a model to study nociception in mouse peripheral afferents using colonic biopsies from patients with abdominal pain (FAPS, CD, UC)
- To understand if specific mediators released from the biopsy can play a role in colonic afferent activation and nociception
- To understand if a therapeutic target exists for future studies to pursue

1.23.2. Electrophysiology

The supernatant was tested on individual serosal layer receptive fields with the control supernatants producing a median change in activation of 0.17 (0.11-0.24) spikes/s^{-1}, FAPS was 0.26 (0.02-0.60) spikes/s^{-1}, CD was 0.37 (0.00-0.59) spikes/s^{-1}, UC was 0.27 (0.01-0.31) ($p<0.8$) . Vehicle response (not shown) was 0.00 (0.00-0.04) spikes/s^{-1}. To understand the translational potential of this afferent firing from CD supernatants, they were also tested on human colonic afferents (N=2, *n*=2) from resected tissue removed at the Royal London Hospital. In one experiment, produced little afferent firing (peak firing rate 0.06 1.32 spikes/s^{-1}). In another experiment, the supernatant produced a robust increase in firing compared with baseline activity (peak firing rate 1.32 spikes/s^{-1}).

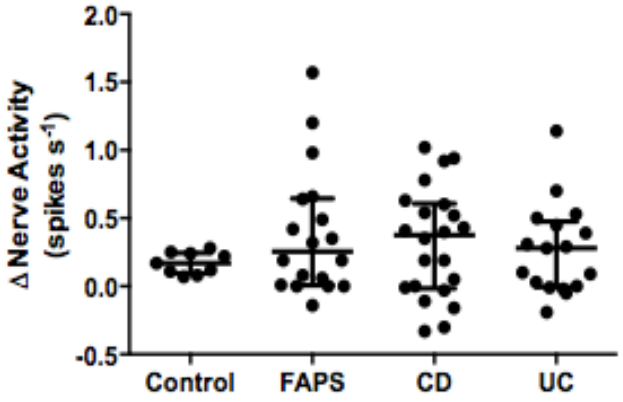
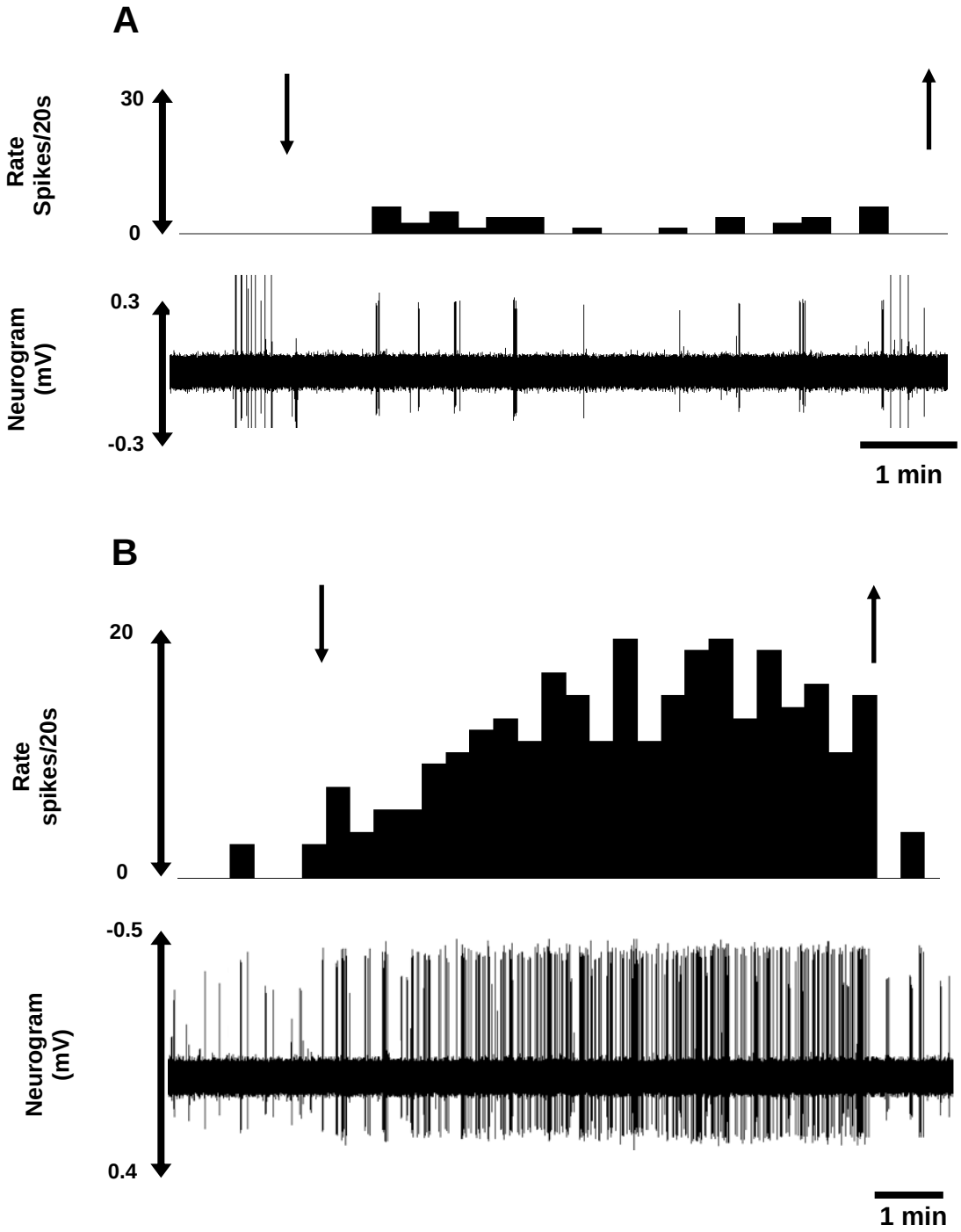


Figure 12. Electrophysiology experiment

Control supernatants gave a small increase in afferent firing from whereas the supernatants from the FAPS, CD, and UC groups had responses that were greater than control. Overall the spread in the afferent responses was much more prominent in patient groups compared with controls suggesting mediators within supernatants from patients are capable of eliciting greater afferent responses. (Control; N=5, *n*=9; FAPS; N=11, *n*=18; CD; N=12, *n*=22; UC; N=10, *n*=16, ANOVA).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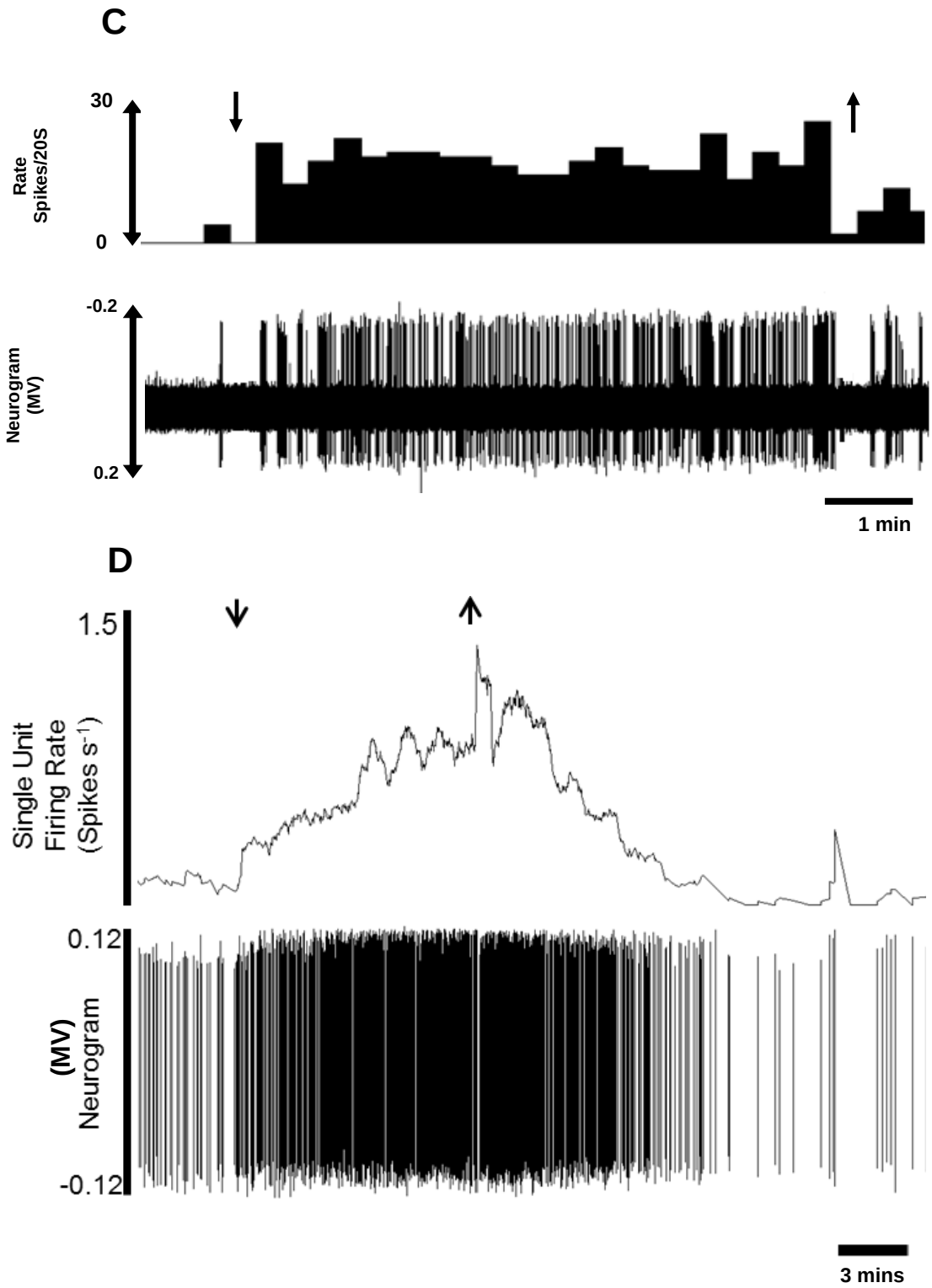
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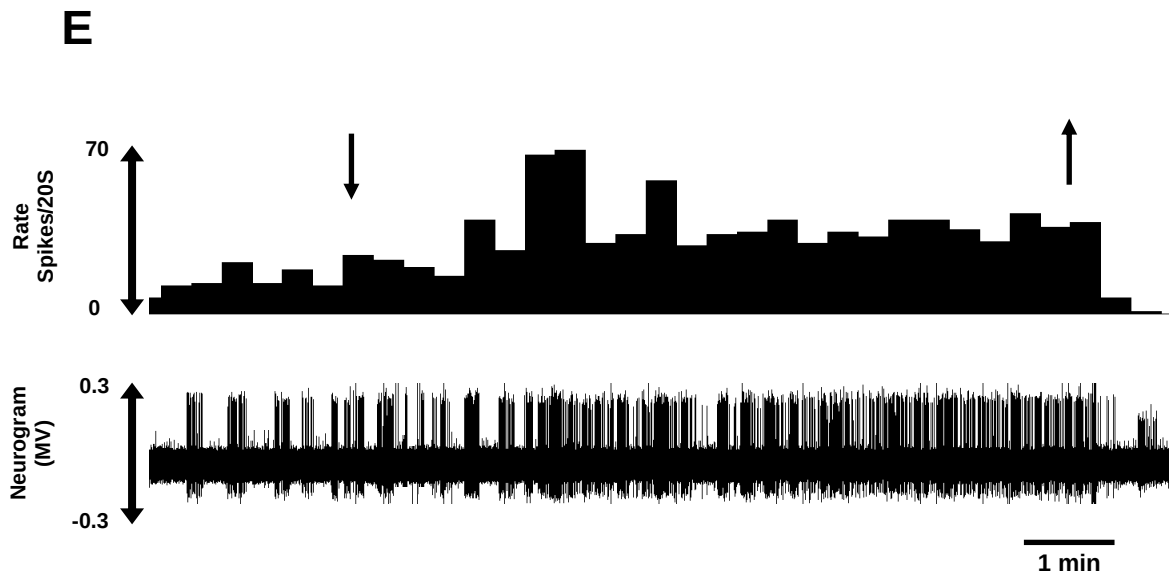


Figure 13. Electrophysiology recordings.

Example traces of afferent responses from **(A)** control supernatants, **(B)** FAPS supernatant **(C)** CD supernatants, **(D)** CD supernatants on a human colon afferent receptive field, **(E)** UC supernatant. The down arrows indicate the application of supernatant and the up arrows indicate the removal thereby showing the spontanteous activity before and after the application of supernatant. Above trace represents the rate histogram of all afferent activity with the bottom trace showing the individual action potentials.

VFH responses

To examine changes in mechanical sensitivity to the receptive field following supernatant incubation, responses to VFh probing was measured pre- and post- incubation. For control supernatants the response showed pre; 5.17 (4.70-9.22) spikes/s-1 compared with post; 4.95 (4.42-9.56) spikes/s-1. FAPS supernatants demonstrated pre; 8.22 (7.73-9.31) spikes/s-1 compared with post; 7.45 (6.70-10.97). CD showed pre; 6.89 (5.89-8.44) spikes/s-1 compared with post; 8.22 (7.00-9.67) and UC showed pre; 8.11 (7.06-8.75) compared with post; 6.95 (6.41-10.33).

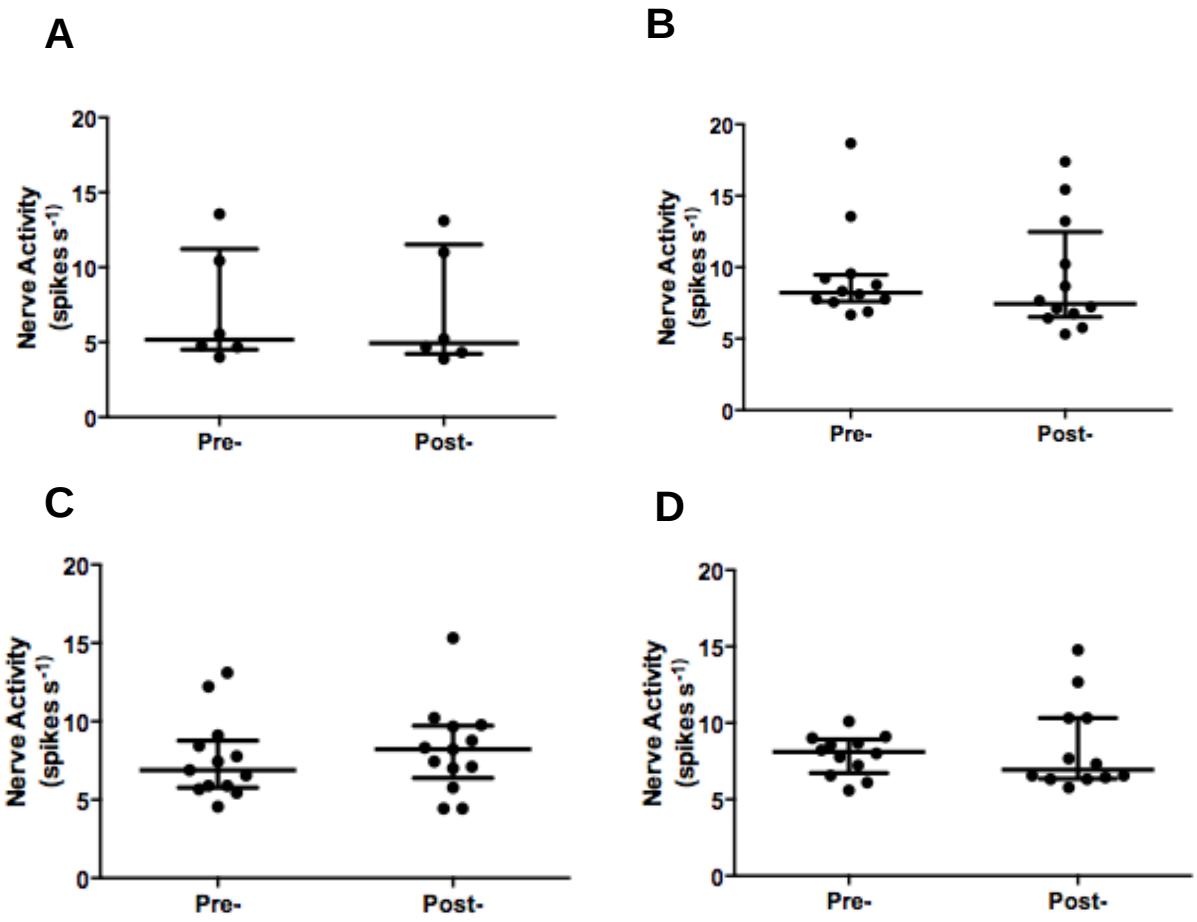


Figure 14. VFh responses

(A) Control biopsy supernatants did not elicit a change in afferent response to blunt probing, nor did **(B)** FAPS supernatants, **(C)** CD supernatants, or **(D)** UC supernatants. (Control; N=5, *n*=5; FAPS; N=9, *n*=12; CD; N=12, *n*=12; UC; N=8, *n*=13, t-test).

1.23.3.Pro-inflammatory cytokines

Protein expression of inflammatory mediators within supernatants were analysed for each patient phenotype. Control and FAPS biopsy supernatants showed extremely low levels of all inflammatory mediators. Consistent with the clinical assessment of no observable bowel inflammation, biopsy supernatant levels of IL-1β, TNFα and IL-6 were below the limit of detection or in the case of IL-8 extremely low in samples from control 1.40 ± 0.77 pg/ml (*n*=6, N=4), and FAPS 1.92 ± 0.81 pg/ml (*n*=11, N=10) patients (figure 11). Similarly, IL-8 biopsy transcript levels were low and comparable between control (*n*=4, N=4) and FAPS

biopsies (*n*=12, N=10) (0.0080 ± 0.0048 vs 0.0050 ± 0.0011, respectively). Levels of IL-8 were significantly greater in CD supernatants than those observed in control supernatants (e.g. 46.18 ± 18.82 pg/ml (*n*=20, N=13), vs 1.40 ± 0.77 pg/ml (*n*=6, N=4), $p<0.05$). Levels of IL-1β (29.9 ± 5.69 pg/ml), IL-6 (42.12 ± 11.08 pg/ml) and TNFα (9.98 ± 4.54 pg/ml) were also raised but these were not found to be significantly different from control supernatants due to the low number of samples reporting cytokine levels above the lower limit of detection. Cytokine levels were measured in UC biopsy supernatants with significantly greater levels of IL-8 being detected in UC samples 89.14 ± 11.79 pg/m (*n*=17, N=10), compared with control supernatants 1.40 ± 0.77 pg/ml (*n*=4, N=5), ($p<0.00001$). All other cytokine levels measured were below the limit of detection.

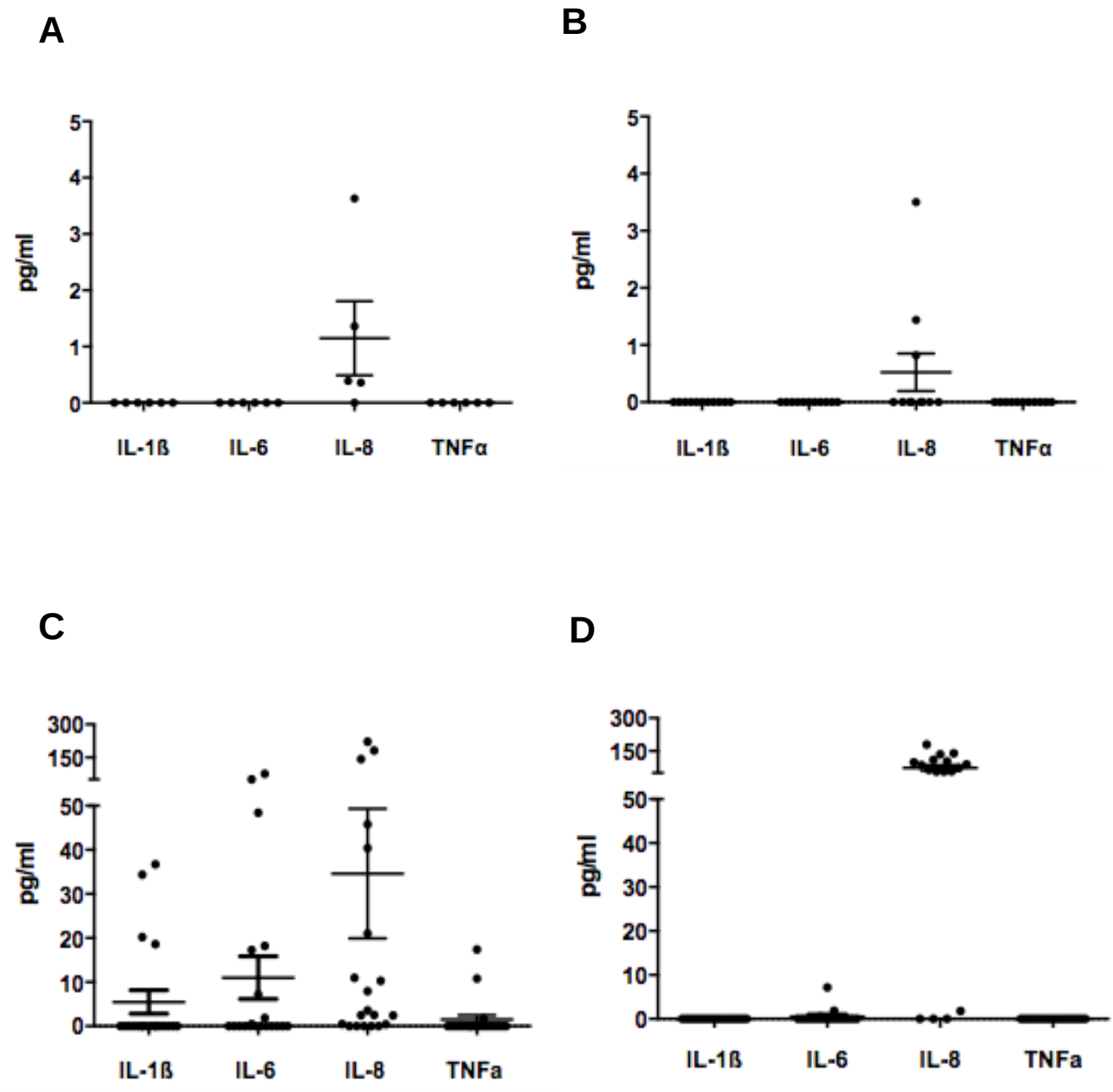


Figure 15. Pro-inflammatory cytokines

(A) No significant changes were observed in cytokines from control supernatants, or **(B)** FAPS supernatants. **(C)** Elevated IL-8 and IL-6 expression was observed in CD supernatants. **(D)** UC supernatants demonstrated increased IL-8 levels but no other pro-inflammatory cytokines**.** (Control; N=5, *n*=5; FAPS; N=10, *n*=12; CD; N=12, *n*=20; UC; N=10, *n*=17).

CHAPTER 2. FUNCTIONAL ABDOMINAL PAIN SYNDROME

2.1. METHODOLOGY

2.1.1. Genotyping trpv4 mice

Genotyping of the mouse colony was performed by Dr Andrew Grant of Kings College London, UK.

2.1.2. DNA extraction from ear

The mouse ear snip was collected into a 0.5ml tube with 30µL PCR lysis buffer added (10% 10x GB buffer, 2% triton X-100, 1% β-mercaptoethanol, 0.002% proteinase K at 19.7mg/ml, 87% dH_2O). Next, the tissue was incubated at 55℃ for 60 minutes, followed by 95℃ for 5 minutes before vortexing for 30 seconds. Next, the tube was centrifuged for 1 minute at 12000rpm and stored at -20℃ until required for PCR.

2.1.3. Performing PCR

The primers for $TRPV_4$ were CATGAAATCTGACCTCTTGTCCC (sense) and TTGTGTACTGTCTGC ACACCAGGC (antisense). The PCR mix per tube included Go-Taq buffer x 5 (4µL), forward primer (10µM, 1µL), reverse primer (10µM, 1µL), dNTP's (10mM, 0.4µL), DNA (1µL), Go-Taq (0.2µL), and dH_2O (12.4µL). Reagents all from Qiagen, UK. Next, the mix was placed in a thermocycler for 30 cycles (94℃ for 30 seconds; 68℃ for 90 seconds; 72℃ for 150 seconds) followed by 72℃ for 10 minutes and kept at 4℃ until collected. The mix was pipetted into a gel plate and electrophoresis occurred for 30 minutes.

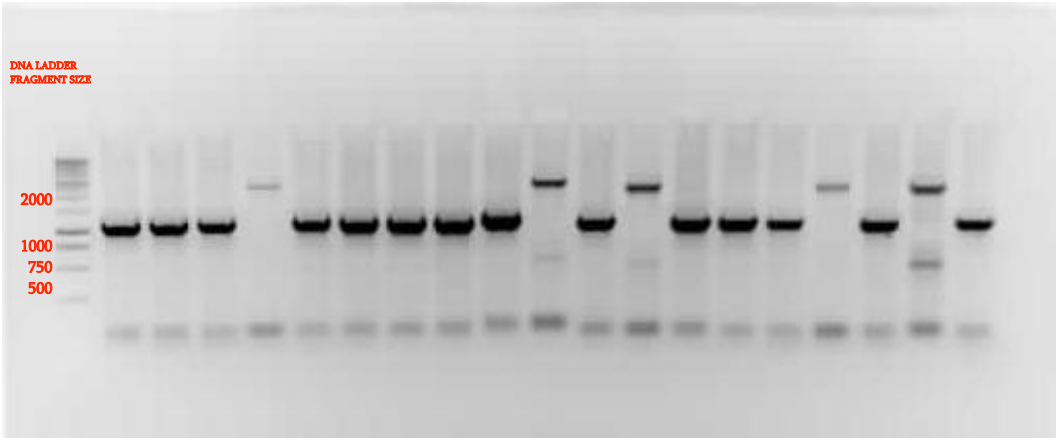


Figure 16. Genotyping $TRPV_4$ mice

The DNA ladder on the left shows the basepairs where $TRPV_4^{-/-}$ are shown at 1.1Kb only, heterozygotes (not seen) would be shown between 1.1-2.1Kb and $TRPV_4^{+/+}$ shown at 2.1Kb only. Above the $TRPV_4^{-/-}$ can be seen at 1.1Kb and $TRPV_4^{+/+}$ can be identified at 2.1Kb.

2.2. RESULTS

Visceral hypersensitivity is a feature associated abdominal pain with adult IBS and is hypothesised to be a result of mast cell infiltration towards nociceptors where prolonged exposure to mast cell mediators such as histamine cause changes in neuronal excitability linked to upregulation of the $TRPV_1$ channel and a coupling to the H_1 receptor. In addition to this mechanism, histamine has also been associated with post-inflammatory visceral hypersensitivity with the H_1 receptor also coupled to the $TRPV_4$ channel. The $TRPV_4$ channel also has important functions as a high-threshold mechanoreceptor within the somatic nervous system and visceral nervous system where pain studies using $TRPV_4^{-/-}$ mice have been able to show a 50% reduction in colonic afferent firing from baseline VFh probing responses.

Therapies which target mast cell degranulation have shown a benefit to IBS patients in reducing abdominal pain episodes. In addition to histamine, 5-HT and tryptase are also capable of eliciting strong afferent activation and have also been linked to pain in rodent models of tissue injury and inflammation. Mast cell tryptase, cathepsin S, and neutrophil elastase, for example, signal through the GPCR PAR_2, and PAR_2-specific agonists can lead to hypersensitivity of afferents in rodents. In a similar coupling as observed with histamine, activation of PAR_2 leads to the downstream activation of $TRPV_1$ and $TRPV_4$ channels through PLC-mediated intracellular pathways and chronic exposure to these proteases is therefore associated with sensitisation from coupling of GPCR's to signal transducer channels. Although this research is promising in adult IBS patients, little is known about the comparative concentrations within paediatric populations suffering with functional abdominal pain. It is reasonable to hypothesise that similar mechanisms may be involved in abdominal pain in FAPS patients but a robust quantitative study is absent.

Hence, this study aims to identify and quantitatively assess the mucosal composition and role of potential mast cell mediators in paediatric FAPS patients. It aims to understand if afferent activation of mouse colonic afferents is in response to specific mast cell mediators with the potential to further investigate specific mediators which correlate with afferent activity with a therapeutic target approach in mind. In addition to mediator quantification and quantitation, this study aims to understand whether the $TRPV_4$ channel may play an integral role in the generation of action potentials from FAPS supernatants, and therefore colonic mucosal immune mediators. This study is the first to attempt to understand the mediator expression and function of the $TRPV_4$ channel in paediatric FAPS patients.

The aims of this chapter are as follows:

- To quantitatively assess mast cell mediators within supernatants from FAPS patients
- To understand if specific mast cell mediators with increased concentrations correlate with afferent activity
- To examine the role of $TRPV_4$ in afferent activation from FAPS patient supernatants to understand if there may be potential for $TRPV_4$ to be targeted for future therapeutic approaches

2.2.2. Quantifying mediator levels

To investigate if mast cell mediators might be responsible for the increased afferent response to FAPS supernatants seen in this study, we examined the biopsy expression of tryptase, histidine decarboxylase (the enzyme responsible for histamine production), and tryptophan hydroxylase I (the enzyme responsible for serotonin production). No significant differences were seen between the expression of these enzymes in control vs FAPS biopsies (e.g. tryptase 0.010 (0.009-0.011) vs 0.011 (0.007-0.014), respectively; histidine decarboxylase $6.4x10^{-5}$ ($5.0x10^{-5}$ – $1.2x10^{-3}$) vs $5.3x10^{-4}$ ($3.0x10^{-5}$ – $9.0x10^{-5}$), respectively, or tryptophan hydroxylase 0.010 (0.009 – 0.011) vs 0.011 (0.007 – 0.014), respectively.

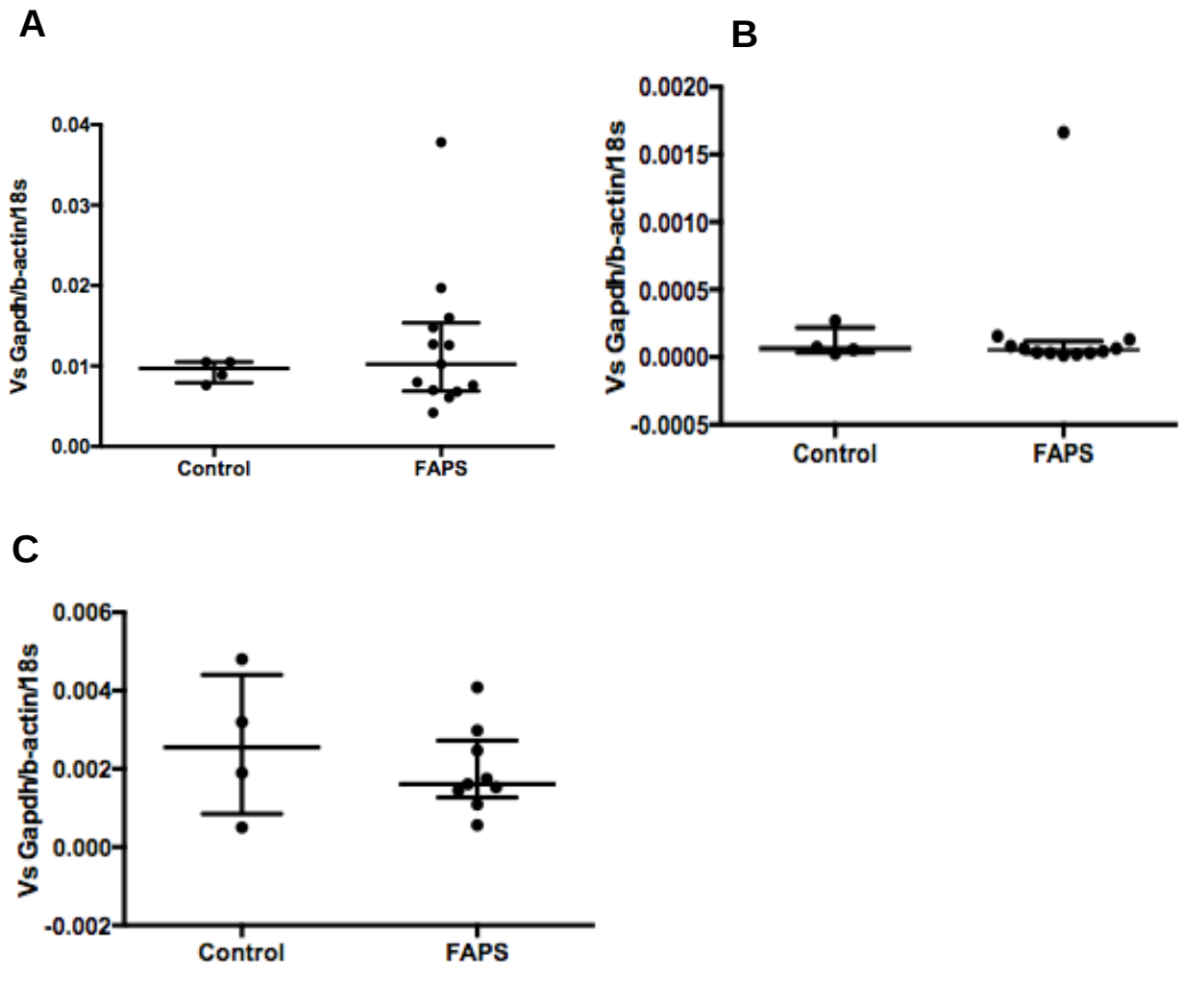


Figure 17. The mRNA levels in FAPS mucosal biopsies.

(A) Mast cell mediators were scrutinised in the FAPS biopsies and compared with control samples for tryptase (Control: *n*=4, N=4; FAPS: *n*=13, N=10), **(B)** histidine decarboxylase, the enzyme involved in the production of histamine (Control: *n*=4, N=4; FAPS: *n*=12, N=10), **(C)** and tryptophan hydroxylase I, the enzyme involved in production of serotonin did not show any difference between the control or FAPS patients (Control: *n*=4, N=4; FAPS: *n*=9, N=10) (Mann-Whitney).

2.2.3. Transient receptor potential vanilloid 4

Next we investigated the mechanism by which FAPS supernatants stimulate serosal nociceptors and so we investigated the possible contribution of $TRPV_4$ to FAPS supernatant mediated afferent activation using tissue from $TRPV_4^{-/-}$ mice. We observed a significant reduction in the afferent response to pooled RAP supernatants in tissue from $TRPV_4^{-/-}$ mice compared with $TRPV_4^{+/+}$ animals (-0.08 ± 0.08 vs 0.27 ± 0.14 spikes/s^{-1} in $TRPV_4^{-/-}$ (*n*=9, N=4) vs $TRPV_4^{+/+}$ (*n*=11, N=5) mice, $p<0.05$) (figure 17). In keeping with this observation the proportion of supernatant responses that could be classified as responders was lower in tissue from $TRPV_4^{-/-}$ mice (22%) compared with tissue from $TRPV_4^{+/+}$ mice (46%) (figure 18). Additionally no change in mechanosensitivity from VFh probing was observed following the application of FAPS pooled supernatants in tissue from $TRPV_4^{+/+}$ or $TRPV_4^{-/-}$ mice (figure 19).

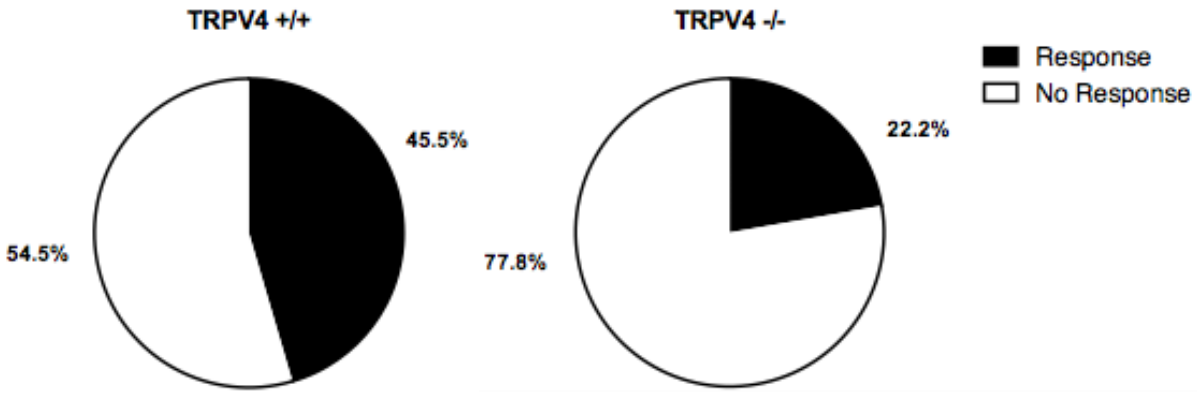


Figure 18. The proportion of afferents that responded to supernatants.

The FAPS supernatants given to serosal receptive fields on $TRPV_4^{-/-}$ (N=4, *n*=9) mice elicited a smaller proportion of responses compared with $TRPV_4^{+/+}$ (N=5, *n*=11) mice. $TRPV_4$, transient receptor potential vanilloid 4 (students t-test was used).

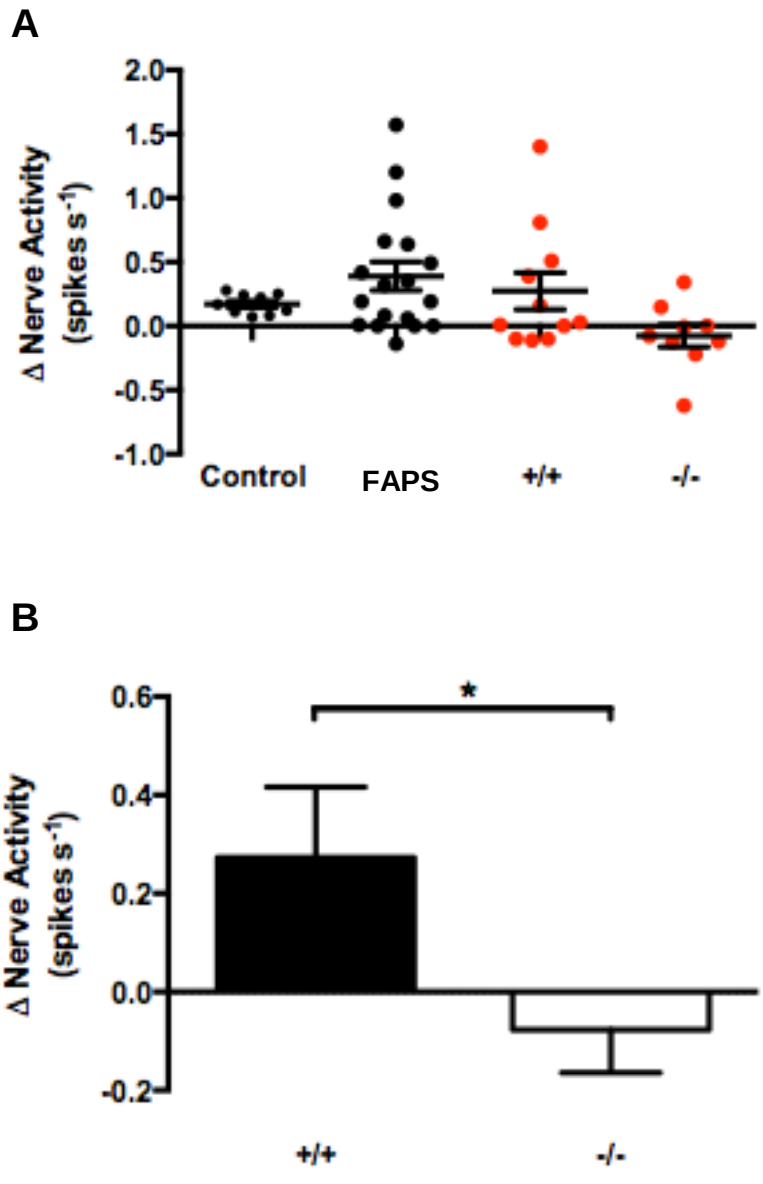


Figure 19. The response to FAPS supernatants in $TRPV_4^{-/-}$ mice.

(A) The responses in control and FAPS groups represent individual patient supernatants. The responses in the $TRPV_4^{+/+}$ and $TRPV_4^{-/-}$ mice show a reduction in the afferent activation in the $TRPV_4^{-/-}$ mice compared to other groups including the $TRPV_4^{+/+}$ mice. **(B)** The responses of only the $TRPV_4^{+/+}$ and $TRPV_4^{-/-}$ mice as a bar chart showing a significant reduction in afferent firing in the $TRPV_4^{-/-}$ mice ($p<0.05$). $TRPV_4$, transient receptor potential vanilloid 4. Bars represent mean ± S.E.M. (Control; N= 5, *n*= 9; FAPS; N= 11, *n*= 19; +/+; N= 5, *n*= 11; -/-; N= 4, *n*= 9; Mann-Whitney).

2.2.4. Mechanical responses

Mechanical responses pre- and post- FAPS supernatant incubation were assessed in $TRPV_4^{+/+}$ and $TRPV_4^{-/-}$ mice. $TRPV_4^{+/+}$ mice showed pre: 8.11 (7.17-10.0) spikes/s^{-1} and post: 8.11 (5.83-9.73) spikes/s^{-1} whereas $TRPV4^{-/-}$ mice showed pre: 8.78 (6.56-11.33) spikes/s^{-1} and post: 8.12 (6.67-9.11) spikes/s^{-1} .

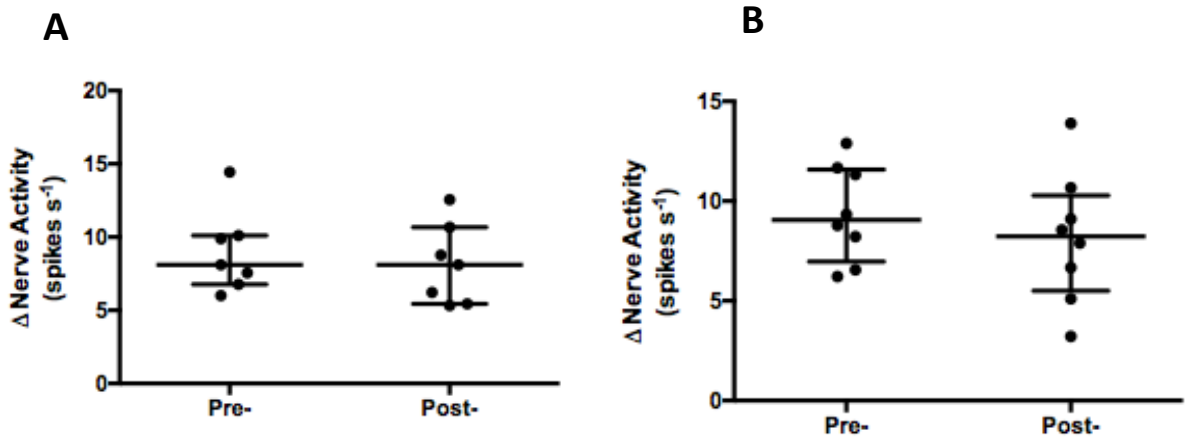


Figure 20. Mechanical responses in $TRPV_4^{+/+}$ and $TRPV_4^{-/-}$ mice.

(A) The probed responses did not significantly change after a 7 minute incubation of FAPS supernatants on $TRPV_4^{+/+}$ mouse serosal layer receptive fields (N=5, *n*=7). **(B)** There was no significant change in probed responses after a 7 minute incubation of FAPS supernatants on $TRPV_4^{-/-}$ mouse receptive fields. (N=4, *n*=7).

2.2.5. SUMMARY OF RESULTS

- ❖ No evidence of mucosal inflammatory processes in biopsies or supernatants was observed
- ❖ Supernatants are capable of robust colonic afferent activation far greater than control supernatants suggesting a pro-nociceptive gut environment
- ❖ No overall change in mechanosensitivity was observed in afferents
- ❖ Colonic afferents from $TRPV_4^{-/-}$ mice produced a reduced response to supernatants suggesting therapeutic basis for a $TRPV_4$ target

2.3. DISCUSSION

2.3.1. Overview

FAPS is a prevalent paediatric gastrointestinal disorder that has a significant impact on the quality of life for the children who suffer from it (Clouse, 2006) (table 1). Despite this our current understanding of its disease pathophysiology is limited, and so treatment efforts are largely directed towards psychological and dietary approaches (figure 1, table 1, 2). A growing body of data supports the presence of pro-nociceptive changes in the bowel of adult patients with the related condition IBS, and a small number of studies suggest the same may be true in paediatric IBS patients (figure 2, 3, table 4). To explore the possible contribution of pro-nociceptive changes to the bowel of paediatric patients with FAPS, we examined the effect of supernatants generated from colonic biopsy samples of patients undergoing investigative endoscopy at the Royal London Hospital in response to treatment refractory, persistent abdominal pain. These patients had not previously displayed symptoms consistent with organic disease, and the absence of a visible organic pathology was confirmed during endoscopy consistent with other clinical tests. This was further confirmed by evaluation of cytokine levels or expression in the supernatant or biopsy samples used in this study.

2.3.2. Afferent response

Mouse colonic afferents were recorded using single-unit electrophysiological techniques whereby the biopsy supernatant could be applied directly to the receptive field to observe any activation of the afferent (figure 13). The incubation buffer was tested and displayed no response on the afferent ending to ensure that any response measured here on out would be assumed to be from the mediators present in the supernatant only, and not influenced by the researcher or the incubation buffer. The control supernatants were added to the receptive fields and elicited a significant response compared with the vehicle. This activation remained extremely consistent throughout all of the control samples and is likely a result of the biopsy procedure itself whereby damage to mucosal cells and nearby blood vessels result in the release of prostaglandins, histamine, potassium, ATP, and bradykinin from the epithelium and endothelial cells, along with local immune cells such as mast cells, macrophages, and cytokines (Bodin, 1998; Reddigari & Kaplan, 1988; Cayrol, 2009; Rock & Kono, 2008). These mediators have previously been shown to stimulate specific receptors on afferent endings and nociceptors have been described which show receptor expression for pro-nociceptive mediators

such as bradykinin, ATP, and PGE2 (Schuelert, 2015; Hockley, 2014; St-Jacques, 2013)

The FAPS supernatants elicited a much greater level of activation than the control supernatants in both the mean firing and maximal response (figure 13). The response profile of the FAPS supernatants also showed a greater variability from the mean response, and this observation was specific only to FAPS responses and statistically significant suggesting that FAPS patients represent a much more heterogeneous population and likely demonstrate a more complex immune environment as shown by the variance in afferent activation. Thus, despite the absence of organic changes within the gut differences between the two groups from this study, there remains a greater capacity to elicit robust sensory afferent response from FAPS supernatants. This observation itself may be an interesting function of patient phenotyping as the patients were not further stratified based on clinical symptoms and pain scores did not reveal relevant similarities to afferent responses. The greater responses may be due to an altered immune environment which may be difficult to detect within this study. Literature suggests increased mast cell infiltration and their proximity to nociceptors is responsible for afferent activation and abdominal pain in adult IBS and the same characteristic could be true for this patient group (Di Nardo, 2014). Mast cell mediators such as histamine and 5-HT are elevated in adult IBS patient mucosal biopsies and these mediators elicit changes in afferent activity through (figure 4) actions depending on H_1 receptor and the 5-HT_3 receptor binding and their coupling to transducer channels such as $TRPV_1$ and $TRPV_4$ (Balemans, 2017; Deiteren. 2014). However, there was a large variation in responses which may be due to mediator expression or variability in the afferent recording technique whereby the afferent itself may not have the required receptor/ion channel groups required to elicit a response from specific supernatants. This is supported by numerous laboratories showing the distributions of receptors and ion channels on small-medium diameter neurons in the gut. For example, with the purinergic receptors, responsible for ATP-specific activation, only 40% of afferent express the P_2X_3 receptor (Brierley, 2005). In light of this variability in afferent subtypes, IBS supernatants have been shown to elicit afferent responses in only 50% of afferents (Buhner, 2009). Although all supernatants were tested twice on the afferents, sub-populations of nociceptors likely play a role in supernatant responses. This is also apparent when we pooled the supernatants together and observed the same characteristic effect that some applications did not elicit an afferent response.

Finally, the supernatants added to the afferent receptive fields may well produce graded responses depending on the mediator involved and concentration within a given supernatant. This may be responsible for the interesting observation that the percentage of supernatants that elicited a robust response above the control (56%) is similar to the proportion of IBS patients who report severe abdominal pain as a recurrent symptom, approximately 54% (Arnott, 2011). This variability in afferent response may therefore support the basis that afferent recordings using patient biopsy supernatants can be used to model abdominal pain in patients although further detailed studies involving precise pain reporting would be needed to investigate this further.

2.3.3. Mechanical responses

Visceral hypersensitivityy is currently the leading hypothesis to explain visceral pain in FGID's such as IBS. It is based around the theory that through peripheral or central mechanisms there is enhanced sensitivity of the pain pathway leading to a lower activation threshold resulting in pain. Interestingly, barostat studies suggest that hypersensitvity is a result of a global change within the gut and not subject to large regional differences meaning that the biopsy itself can not be assumed as a cause of afferent firing variability (Ginkel, 2001; Bouin, 2002).

Barostat studies in IBS patients observe reduced pain thresholds and this is supported by functional brain imaging showing increased activation in pain processing regions (Bouin, 2002). To understand if the FAPS supernatants in this study could generate similar mechanical sensitisation of the afferents we looked at VFh probed responses before and after the application of the supernatant (figure 14). Changes in VFh responses were not observed overall in this study, however, it is possible that the VFh probing may not have the sensitivity to detect small changes to the neuronal excitability. Although VFh probing can be used as a reliable measure for changing mechanical sensitivities in the presence of sensitising mediators such as bradykinin, it is likely to be susceptible to mediator concentrations and afferent subtypes involved (Brierley, 2004). It is intriguing that the mechanical probing responses seem minimal compared to what would be expected from well defined clinical visceral hypersensitivity. For example, the effect size observed here is in disagreement to studies by Hughes and colleagues which produced large changes in VFh responses when IBS-D

supernatants were applied to colonic afferents (Hughes, 2009; Hughes, 2013). Studies support evidence that shows how adult IBS supernatants from mucosal biopsies induce visceral hypersensitivity to CRD and blunt probing which has been shown to result from influences by histamine and tryptase via intracellular phosphorylation of TRP channels (Barabara, 2007; Crouzet, 2013; Balemans, 2017). Although small increases in the expression of histamine and tryptase in this study were observed, expression was not significantly elevated which could account for differences in results reported in literature. Importantly, as FAPS patients were used in this study as opposed to stratified IBS patients, this may explain differences in findings, as there are no current studies which aim to understand probing responses from FAPS supernatants.

2.3.4. Pro-nociceptive mast cell mediators

Elevated mast cells and their proximity to sensory nerves is thought to play a major role in IBS abdominal pain (Barbara, 2007; Di Nardo, 2014). Mast cells are responsible for significant increase in histamine, tryptase, and PGE_2, in IBS patients when compared to healthy controls (Henderson, 2012; Taylor, 2010; Buhner, 2009). Based on the concept that mast cell mediators may be responsible for neuronal hypersensitivity and increased visceral pain in IBS patients, the same could be hypothesised for other FGID's such as FAPS, therefore mediator levels were quantified (figure 17). As histamine, 5-HT, and tryptase, all release by mast cells, have been shown to elicit afferent firing, these mediators were assessed. In this study, there were no significant increases in transcriptional expression of histidine decarboxylase and tryptophan hydroxylase I (indirectly measuring histamine and 5-HT, respectively) or tryptase, although FAPS samples did show a much greater peak range of tryptase compared with control samples. To date, this is the first have published the expression of histidine decarboxylase and tryptophan hydroxylase I in FAPS patients although the expression of tryptophan hydroxylase I in adult IBS patients has previously been reported in rectum and colonic mucosa (Kerckhoffs, 2011). As mRNA expression gives no evidence to the proximity of functional proteins to endogenous neurons, or the true protein expression, it is possible that imaging techniques from whole tissue samples may have revealed more and this is a consideration for the future.

The greater range of tryptase expression observed in this study could be an indication of a change in production which is consistent with literature which suggests that increased tryptase may have a direct role

in visceral pain (Cenac, 2007; Roman, 2014; Schwartz, 2015). However, tryptase expression in this study was not significantly elevated and it is important to note from this study that the data could be vulnerable to a type II error due to a potentially underpowered control group making it difficult to detect smaller changes in mediator levels between the two groups, and further investigation would be warranted.

2.3.5. Transient receptor potential channel

Although analysis of the biopsies did not reveal elevated expression of any potential mediators involved in nociception, a therapeutic approach to reducing visceral pain in patients remains an important target. $TRPV_4$ is a member of the transient receptor potential family of ion channels. It is a non-selective cation channel with a preference to calcium ions. $TRPV_4$ is expressed on colonic afferents and blockade has been shown to reduce pain behaviours (Sipe, 2008; Cenac, 2008; Brierley, 2008; Fichna, 2012). Based on our current understanding of this channel, the FAPS supernatants were added to colonic afferents from $TRPV_4^{+/+}$ and $TRPV_4^{-/-}$ mice (figure 16). The responses seen in $TRPV_4^{+/+}$ mice were similar to the responses originally seen in the C57BL/6 WT mice. Importantly, this response was much greater than those observed in the $TRPV_4^{-/-}$ mice (figure 18, 19), suggesting that the absence of $TRPV_4$ significantly reduced the ability of the supernatants to elicit a strong afferent activation. This is also evidenced when looking at the proportion of responses in the $TRPV_4^{+/+}$ mice compared with the $TRPV_4^{-/-}$ mice, in which responses were recorded in 46% compared with only 22% of afferents, respectively. The $TRPV_4$ channel has been linked to pain mechanisms largely due to its coupling with the PAR's whereby the activation of PAR_2 on afferent endings leads to an increase in intracellular calcium triggering PLC-β and PLA_2 pathways which result in the opening of $TRPV_4$ (and also $TRPV_1$ and $TRPA_1$ via PKA and PKC) to further increase calcium depolarising the afferent ending, potentially leading to the release of CGRP which binds to mast cell G-protein coupled receptors to promote the release of histamine, another pro-nociceptive mediator to further stimulate the afferent (Sipe, 2008; Zhao, 2015; Grant, 2007; Poole, 2013; Grace, 2014). $TRPV_4$ has also been shown to play an important role in mechanical sensitivity during hyperalgesia and inflammation and although literature has indicated that $TRPV_4^{-/-}$ mice have diminished mechanical sensitivity, this was not observed in this study (Cenac, 2008; Brierley, 2008; Fichna, 2012). Mechanical pain studies in $TRPV_4^{-/-}$ mice demonstrate a 50% reduction in colonic afferent

responses to VFh probing but this was not observed in this study *figure 20) (Meuller-Tribbensee, 2015; Sipe, 2008; Brierley, 2018).

This result remains unclear but may be linked with the experimental design of each study whereby different mechanical testing such as CRD or a wider range of VFh probes could provide more detailed observations, and this would be a consideration for future studies. It is possible that the use of 1g VFh meant that only sub-maximal pressure was applied and that threshold changes to serosal afferents in $TRPV_4^{-/-}$ would only become apparent at higher probing weights suggesting that $TRPV_4$ plays a more substantial role when mechanical stimuli are at the upper end of noxious levels and this is a limitation of the VFh probing in this study. Taken together, this study compliments current literature suggesting blocking the $TRPV_4$ ion channel may be an effective way of reducing nociception and potentially pain in FAPS paediatric patients.

2.3.6. Conclusion

The findings from this study show that pro-nociceptive changes have occurred in the bowel of a subset of patients with functional abdominal pain undergoing biopsy at the Royal London Hospital as part of their clinical evaluation. This finding suggests that an underlying pathology is responsible for the presence of abdominal pain in these patients, namely peripheral sensitisation of visceral nociceptors. As a consequence, treatments that target visceral nociceptor activation could provide effective relief from abdominal pain in this subset of patients and hence effective treatment of FAPS.

CHAPTER 3: CROHN'S DISEASE

3.1. METHODOLOGY

3.1.1. Activation of MMP-12 using APMA

In order to activate MMP-12 it first must be incubated in a solution of APMA. The preparation of APMA required that a stock solution was made which would be valid for one week by which diluted solutions would be made. First, 3.5 mg of APMA (Sigma-Aldrich, A9563) was dissolved in 1 ml of 0.1M NaOH to prepare 3.5mg/ml solution at 10mM. This stock was kept at 4℃ for up to 1 week. Further dilution of APMA is done on the day of experimentation to ensure maximum stability of the compound. The 10mM stock was diluted to 2.5mM stock using Krebs buffer and the pH adjusted to pH 7 using 0.1M HCl. This 2.5mM APMA was added to MMP-12 in a 1:9 ratio and was incubated for 2 hours at 37℃.

3.1.2. Application of MMP's on receptive fields

The concentration of MMP-12 was found by dose ranging studies from calcium imaging techniques in mouse DRG (see appendix 4) and replicated on receptive fields in electrophysiology studies. To understand the exogenous effects of MMP-12 (also MMP-9) it was added directly to receptive fields. First, a metal ring was placed over the receptive field. A 3 minute baseline of afferent activity was recorded. Next, the buffer was aspirated from the ring and 200µL of pre-warmed (35℃) MMP-12 protein or catalytic domain (50nM) , or MMP-9 catalytic domain (25nM), was added for 7 minutes. After this time, the ring and MMP was removed. Probing with 1g VFh occurred pre- and post-application of MMP and is described in chapter 2. Human serosal receptive field application used 100nM MMP-12 catalytic domain (7 minutes) and 75nM MMP-9 catalytic domain. Vehicle for this application was Krebs buffer.

3.1.3. Chemical sensitisation by MMP-12

To understand whether or not the MMP-12 could indirectly affect afferent firing in the presence of nociceptive mediators, an inflammatory soup (I.S) (1µM bradykinin, 1mM ATP, 10µM histamine, 10µM PGE_2, and 10µM 5-HT) was added to receptive fields. First, a metal ring was placed over the receptive field. A 3 minute baseline of afferent activity was recorded. Next, the buffer was aspirated from the ring and 200µL of pre-warmed (35℃) I.S was added to the ring for 5 minutes. After this time, the I.S and ring were removed. Next, a 25 minute washout period was recorded. The metal ring was then placed over the same receptive

field for 3 minutes. Next, the buffer was aspirated from the ring and pre-warmed (35℃) MMP-12 catalytic domain (50nM) was added for 4 minutes. Next, the MMP-12 was removed and the inflammatory soup was added for a further 5 minutes. Next, the inflammatory soup and the metal ring were removed and the afferent activation of both incubations were analysed and compared.

3.1.4. Human Tissue collection

Human whole tissue collection (segments ofresected colon rather than biopsy tissue) was performed by Dr. Cian McGuire. All human tissue was collected and used with the approval of the East London and the City HA Local Research Ethics Committee (NREC 10/H0703/71). Resected human colon was collected after written consent from patients undergoing elective surgery as part of their standard clinical treatment at the Barts Health NHS Trust (London, UK). All tissues were obtained from a histopathologist following clinical examination. Macroscopically normal tissues were obtained from patients with non-obstructive tumours. Tissues were used immediately after collection and followed the same methodology as mouse electrophysiology recordings.

3.2. RESULTS

Abdominal pain is one of the most common symptoms of GI diseases, accounting for over millions outpatient visits each year worldwide. The UK IBD audit found that over 80% of IBD paediatric patients suffered from abdominal pain with more than half of these patients reporting severe pain. Identical numbers have been observed in adults suggesting that the abdominal pain will persist into adulthood. Currently, animal models such as TNBS and DSS attempt to mimic inflammatory processes and therefore inflammatory pain although to date there is no approach which accurately models pain reported in patients to *in vitro* or *in vivo* models of pain. By utilising mucosal biopsy supernatants and electrophysiological recording techniques, this study attempts to understand if pain can be modelled accurately in an investigation into nociception. In addition to this, it is important to understand that approximately half of patients who undergo treatment for IBD, inclusive of targeted abdominal pain treatments, still report high levels of pain and current therapies offer strong anti-inflammatory control but this does not necessarily translate to reduced pain levels. This is evident in patients who etner into clinical remission but still experience episodes of severe abdominal pain. It is therefore, understandable to propose that inflammatory mediators and the sensitising effects of these mediators on visceral nociceptors persist regardless of current anti-inflammatory treatment and more targeted approaches may be necessary in the future. Currently, no study has assessed colonic afferent activity and inflammatory mediator concentrations to understand this therapeutic window.

A large area of investigation in IBD pain understandably focuses on pro-inflammatory cytokine levels such as IL-1β, TNFα, IL-6, and IL-8. As involvement in maintaining chronic bowel inflammation is also a feature of IL-1β, studies showing mice with depleted ICE (IL-1β-converting enzyme) have reported a protective phenotype from DSS, suggesting that along with the reported importance of IL-1β in the initiation and maintenance of peripheral sensitisation during inflammation, there remains a strong possibility that this cytokine could be a therapeutic target in visceral pain. IL-6 stimulates visceral nociceptors and increased responses to mechanical stimuli have been observed in the presence of TNFα. In addition, IL-8 is a neutrophil chemoattractant that attracts and activates neutrophils during mucosal inflammation to enhance their migration from blood into tissue which has been studied in detail for many years and since its expression was first observed in active IBD mucosal biopsies. Current therapeutic approaches in clinical use and currently being investigated have shown some promising results, the most notable, are the anti-TNFα

immunotherapies, however, as abdominal pain is reported in patients in clinical remission, there is a justification in an approach to understand whether there are further mediators present that are contributing to abdominal pain. MMP's have been mildly suggested to be associated with pain however there is no study which looks to understand their direct influence on afferent activation and nociception. MMP's are a family of proteases that degrade the extracellular matrix (ECM) digesting denatured collagens, in particular cartilage MMP's have been linked to the pathophysiology of IBD, and increased production is observed in CD and UC patients compared with control groups and techniques enabling specific labelling of areas of the colon have shown strong MMP expression in the mucosal epithelium and lamina propria, in both adult and paediatric IBD patients. MMP's such as MMP-9 and MMP-12 have been shown to be released from macrophages at sites of inflammation and MMP-12 in particular has been implicated in the pathophysiology of several disorders such as asthma, COPD, and IBD, where inflammation and fibrosis are dominant clinical features.

This study aims to therefore understand whether further mediators present within the colonic mucosa of CD patients are capable of stimulating nociceptors and contributing to abdominal pain. In particular, MMP expression will be studied and its expression will be assessed in relation to afferent activation to understand if any causal link exists.

3.2.1. The aims of this chapter are as follows:

- To model patient abdominal pain with in vitro electrophysiology based on supernatants generated from colonic mucosal biopsies
- To understand if there is a group of mediators that are expressed which could be exploiting a deficit in current clinical treatment which are responsible for afferent activation
- To understand the influence of MMP's in nociception
- To identify likely sources of mediators if a correlation between inflammatory mediator and afferent response is observed

3.2.2. Afferent activity may act as a representative pain marker

When the afferent firing rates for the CD group were compared with the mean pain scores a striking trend appeared. The supernatants that generated the lowest levels of afferent firing came from patients reporting the lowest pain scores for both the day (r^2 = 0.07; p<0.40) and month (r^2 = 0.008; p<0.42) preceding biopsy surgery (figure 21). The greater the afferent activity, the higher the mean pain scores for both the day and month preceding biopsy surgery.

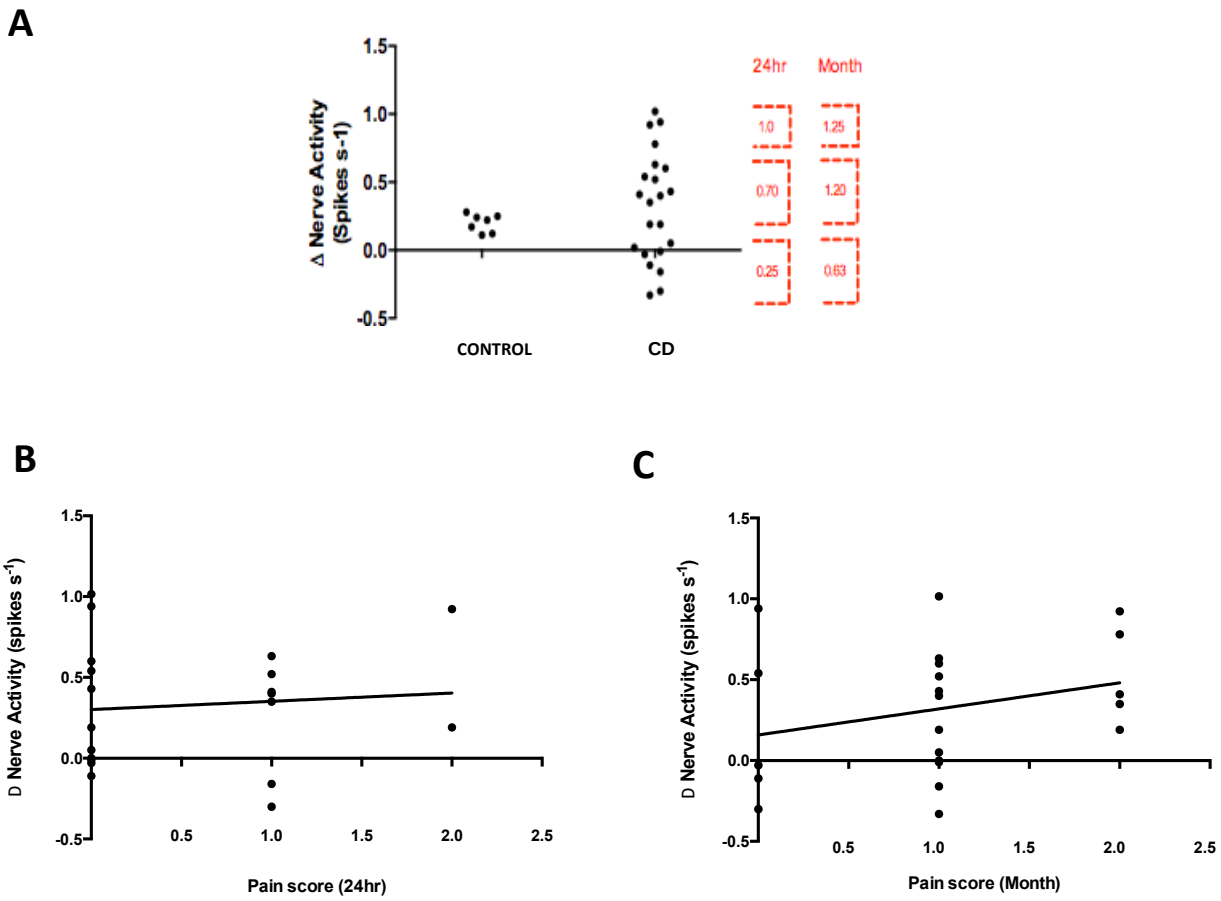


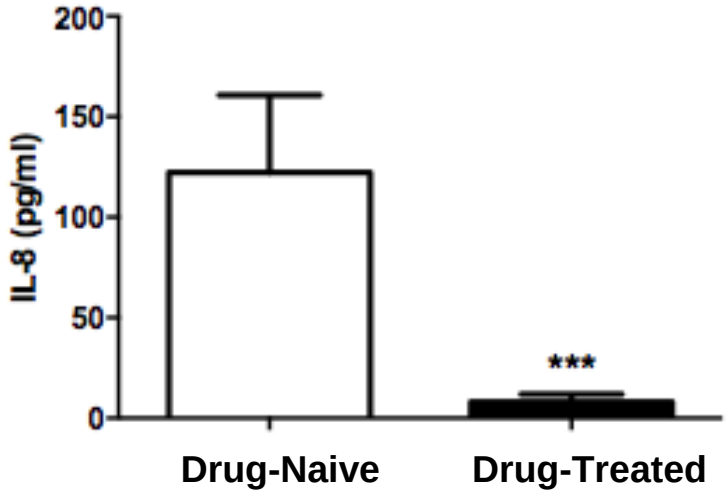
Figure 21. Afferent activity may represent patient pain scores

(A) In CD patients, pain scores taken on the day of surgery, and the pain scores given for the previous month relate to the level of afferent activity elicited from the patient supernatants. The pain scores represent the mean for all patient biopsies within each band. Vas pain scores from 0-3 (no pain, mild, moderate, severe) (control; N=5, *n*=7; CD; N=12, *n*=22). **(B)** Correlation between pain score on the day of the biopsy and afferent response, **(C)** correlation between pain score in the previous month and afferent rersponse. (Linear regression, ANOVA).

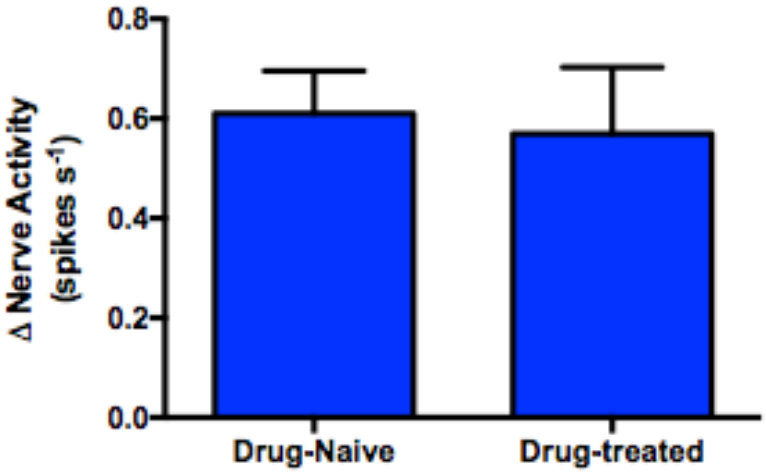
3.2.3. Drug treated patients retain ability to activate colonic afferents

Within the Crohn's disease patients, the ability to stratify patients based on the treatment history allowed analysis to be conducted on the afferent response from each patient group (figure 29). Using protein expression of IL-8 as a marker for local colonic inflammation, the drug-treatment group (122.22 ± 38.54 pg/ml) showed a significant reduction ($p<0.01$) compared with drug-naive CD patients (8.12 ± 3.78 pg/ml). When the 'responders' from the two treatment groups were compared with regards to afferent activation, there remained similar levels of firing (drug-treated; 0.61 ± 0.09 spikes/s^{-1}; drug-naive; 0.57 ± 0.13 spikes/s^{-1}). Interestingly, the proportion of afferent responses was reduced in the drug-treated group (42%) compared with the drug-naive group (80%) where this difference was marginally above statistical significance ($p=0.07$).

A


200
150
100
50
0
IL-8 (pg/ml)
***
Drug-Naive
Drug-Treated

B


0.8
0.6
0.4
0.2
0.0
Δ Nerve Activity
(spikes s-1)
Drug-Naive
Drug-treated

C

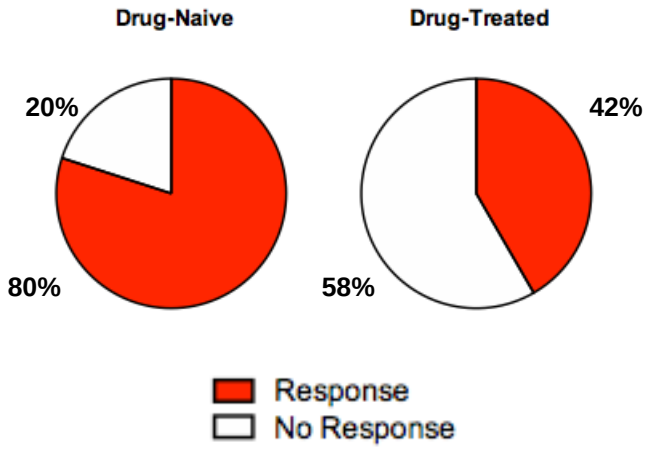

Drug-Naive
Drug-Treated
20%
80%
58%
42%
Response
No Response

Figure 22. A reduced inflammatory environment retains afferent activity

(A) The level of IL-8 which serves as a marker for colonic inflammation is significantly reduced compared with drug-naive CD patients ($p<0.01$). **(B)** This reduction in IL-8 does not effect the ability of supernatants to elicit robust levels of afferent activation. **(C)** Although the afferent response was similar between the treatment groups, the proportion of responses was reduced in patients who were receiving treatment. (N= 6 , *n*= 6; t test).

3.2.4. Biopsy transcript expression

To help determine which mediators might be responsible for the change in colonic afferent activity transcript levels of pro-inflammatory mediator were measured in biopsies samples (figure 23). Median ranscript levels for IL-1β, IL-6, and TNFα were all found to be significantly increased in CD biopsies when compared with controls $7.4x10^{-3}$ ($2.1x10^{-3}$ – 0.02) vs (0.0016 ($7.0x10^{-4}$ – $3.0x10^{-3}$), $p<0.05$; $2.0x10^{-4}$ ($7.8x10^{-5}$ – $3.8x10^{-4}$) vs $7.0x10^{-5}$ ($2.0x10^{-5}$ - $8.0x10^{-5}$) $p<0.01$; $5.5x10^{-4}$ ($2.8x10^{-4}$ – $1.0x10^{-3}$) vs $1.7x10^{-4}$ ($1.0x10^{-4}$ – $3.1x10^{-4}$) $p<0.01$, respectively, and IL-8 transcripts were elevated ($8.6x10^{-3}$ ($2.3x10^{-3}$ – $2.3x10^{-2}$) vs 0.038 (0.035 – 0.054). In addition, mean transcript levels for all cytokines were greater in biopsy samples from drug-naive compared with drug-treated CD patients (IL-1β, 0.013 ± 0.009 vs 0.047 ± 0.021; IL-6, 0.00022 ± 9.3 x 10^{-5} vs 0.00099 ± 0.00046; IL-8, 0.016 ± 0.008 vs 0.055 ± 0.030, respectively) although there was only a limited increase in the TNFα mRNA (0.00084 ± 0.00039 vs 0.00097 ± 0.00032, respectively). No correlation between their transcript levels and afferent firing rate was observed (IL-1β; $p=0.12$, $r^2=0.015$; IL-6; $p=0.17$, $r^2=0.006$; TNFα; $p=0.74$, $r^2=0.010$; IL-8, $p=0.28$, $r^2=0.005$).

The COX-1 transcripts showed a slightly decreased expression to control biopsies (0.0046 ± 0.0006 vs 0.0069 ± 0.0020, respectively). The COX-2 expression was greatly increased in CD compared with control biopsies (0.0012 ± 0.0003 vs 0.00037 ± 0.00011, respectively) with a greater expression in drug-naive patients than drug-treated, compared with controls (COX-1; 0.0037 ± 0.0006 vs 0.0051 ± 0.0009; COX-2; 0.00096 ± 0.00047 vs 0.0015 ± 0.0006, respectively). When the COX-1 expression was correlated with afferent firing, no significant correlation was found ($p=0.97$, $r^2=0.001$). Similarly when the COX-2 expression was correlated with afferent firing, no significant correlation was seen ($p=0.19$, $r^2=0.03$).

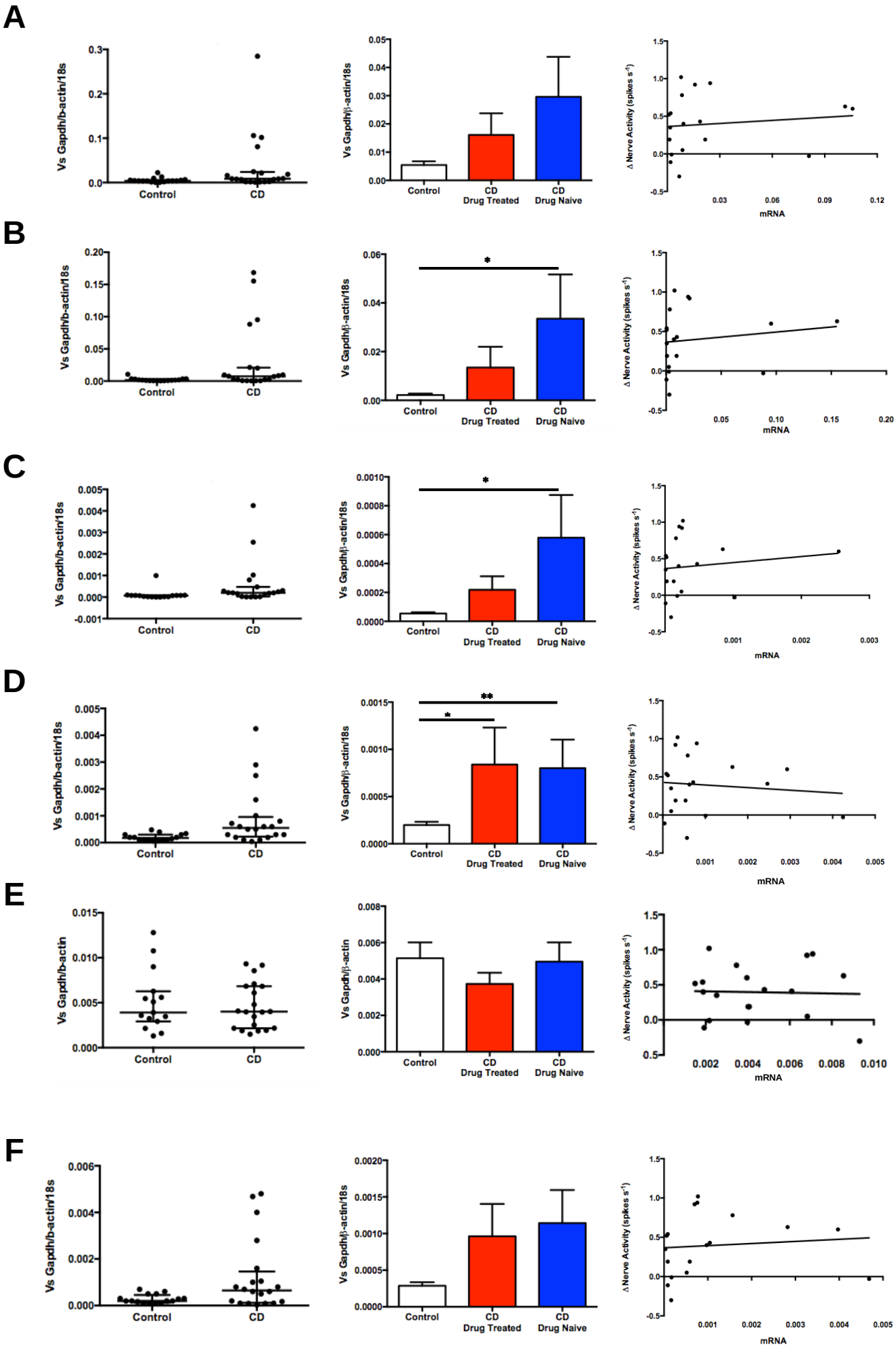

A
B
C
D
E
F
Vs Gapdh/b-actin/18s
Vs Gapdh/β-actin/18s
Vs Gapdh/b-actin
Control
CD
CD Drug Treated
CD Drug Naive
Δ Nerve Activity (spikes s⁻¹)
mRNA
*
**

Figure 23. The transcript levels of inflammatory mediators.

The mRNA levels in CD biopsies of **(A)** IL-8, **(B)** IL-1β, **(C)** IL-6, **(D)** TNFα, **(E)** COX-1 and **(F)** COX-2, compared with control patients (left column). A significant increase in transcript levels of IL-1β, IL-6, and TNFα was observed compared with control biopsies. Patients who were drug-naive exhibited increased levels of IL-8, IL-1β, IL-6 and COX-2 mRNA (middle column). No correlations between inflammatory mediators or their enzymes, and afferent firing rates was observed (right column). Expression data shows median (IQR) whereas drug treatment data shows mean ± S.E.M values where* $p<0.05$; ** $p<0.01$. (Control; N= 16, *n*= 20; CD; N=12, *n*= 18; Mann-Whitney, t-test, linear regression, ANOVA).

3.2.5. MMP'S

As inflammatory mediators such as pro-inflammatory cytokines and classical inflammatory mediators such as prostaglandins, histamine, and 5-HT, did not appear to heavily influence the supernatant's ability to activate the afferent, other mediators were assessed. Matrix metalloproteinases have been reported to be elevated in IBD in a number of studies. Although there is some literature focused on MMP levels in paediatrics, much less is known about MMP levels in colonic biopsies. Here, the MMP-1, MMP-3, MMP-9, MMP-12, MMP-19, and TIMP-1 mRNA levels were analysed (figure 33).

3.2.5.1. MMP-1

The MMP-1 expression was significantly increased in CD compared with control biopsies (0.0051 ± 0.0030 vs $0.0001 \pm 3.8 \times 10^{-5}$, respectively, $p<0.05$), with a greater expression in drug-naive patients than drug-treated (0.010 ± 0.05 vs 0.0028 ± 0.0020, respectively). When the MMP-1 expression was correlated with afferent firing, a significant correlation was found ($p<0.05$, $r^2=0.01$).

3.2.5.2. MMP-3

The MMP-3 expression was significantly increased in CD compared with control biopsies (0.013 ± 0.005 vs $0.0001 \pm 4.6\ 10^{-5}$, respectively, $p<0.001$), with a greater expression in drug-naive patients than drug-treated (0.016 ± 0.008 vs 0.011 ± 0.008, respectively). When the MMP-3 expression was correlated with afferent

firing, no significant correlation was found (p=0.35, r^2=0.17).

3.2.5.3. MMP-9

Again, the MMP-9 expression was significantly increased in CD compared with control biopsies (0.0078 ± 0.001 vs 0.0018 ± 0.0006, p<0.0001, respectively), with a greater expression in drug-naive patients than drug-treated (0.0081 ± 0.0022 vs 0.0074 ± 0.0013, respectively). When the MMP-9 expression was correlated with afferent firing, no significant correlation was found (p=0.94, r^2=0.0003).

3.2.5.4. MMP-12

When the MMP-12 expression was significantly increased in CD compared with control biopsies (0.0014 ± 0.0002 vs 0.0006 ± 0.0002, p<0.01, respectively), with a greater expression in drug-naive patients than drug-treated (0.0016 ± 4.4 10^{-4} vs 0.0022 ± 0.0006, respectively). When the MMP-12 expression was correlated with afferent firing the data follows a significant linear trend (p<0.01, r^2= 0.44).

3.2.5.5. MMP-19

The MMP-19 expression was increased in CD compared with control biopsies (0.0014 ± 0.0002 vs 0.0006 ± 0.0001, respectively, p<0.01), with a greater expression in drug-naive patients than drug-treated (0.0016 ± 4.3 10^{-4} vs 0.0012 ± 0.0002, respectively). When the MMP-19 expression was correlated with afferent firing, no significant correlation was found (p=0.17, r^2=0.11).

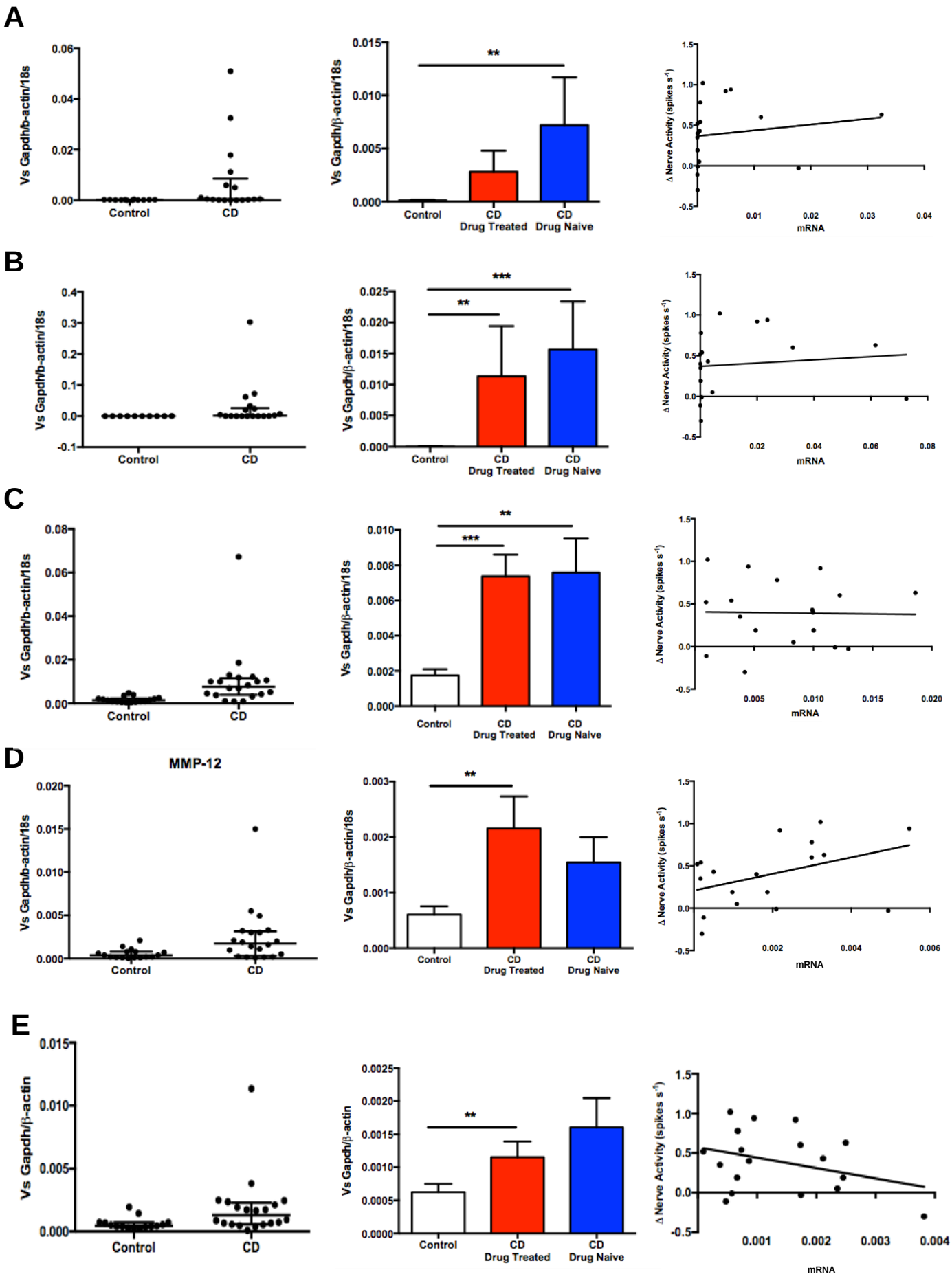

A
Vs Gapdh/b-actin/18s
0.06
0.04
0.02
0.00
Control
CD
Vs Gapdh/β-actin/18s
0.015
0.010
0.005
0.000
**
Control
CD
Drug Treated
CD
Drug Naive
Δ Nerve Activity (spikes s-1)
1.5
1.0
0.5
0.0
-0.5
0.01
0.02
0.03
0.04
mRNA
B
Vs Gapdh/b-actin/18s
0.4
0.3
0.2
0.1
0.0
-0.1
Control
CD
Vs Gapdh/β-actin/18s
0.025
0.020
0.015
0.010
0.005
0.000
***
**
Control
CD
Drug Treated
CD
Drug Naive
Δ Nerve Activity (spikes s-1)
1.5
1.0
0.5
0.0
-0.5
0.02
0.04
0.06
0.08
mRNA
C
Vs Gapdh/b-actin/18s
0.08
0.06
0.04
0.02
0.00
Control
CD
Vs Gapdh/β-actin/18s
0.010
0.008
0.006
0.004
0.002
0.000
**
***
Control
CD
Drug Treated
CD
Drug Naive
Δ Nerve Activity (spikes s-1)
1.5
1.0
0.5
0.0
-0.5
0.005
0.010
0.015
0.020
mRNA
D
MMP-12
Vs Gapdh/b-actin/18s
0.020
0.015
0.010
0.005
0.000
Control
CD
Vs Gapdh/β-actin/18s
0.003
0.002
0.001
0.000
**
Control
CD
Drug Treated
CD
Drug Naive
Δ Nerve Activity (spikes s-1)
1.5
1.0
0.5
0.0
-0.5
0.002
0.004
0.006
mRNA
E
Vs Gapdh/β-actin
0.015
0.010
0.005
0.000
Control
CD
Vs Gapdh/β-actin
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
**
Control
CD
Drug Treated
CD
Drug Naive
Δ Nerve Activity (spikes s-1)
1.5
1.0
0.5
0.0
-0.5
0.001
0.002
0.003
0.004
mRNA

Figure 24. The transcript levels of MMP's in Crohn's disease biopsies.

The mRNA levels in CD biopsies of **(A)** MMP-1, **(B)** MMP-3, **(C)** MMP-9, **(D)** MMP-12, and **(E)** MMP-19 compared with control patients. A significant increase in MMP's and TIMP-1 was observed in disease. This increase was seen to be much higher in patients who were drug-naive compared with patients already receiving treatments. MMP-12 shows a positive correlation towards MMP expression and afferent nerve firing. Bars in expression data represent median (IQR) whereas bar in drug treatment data represent mean ± S.E.M values where * $p<0.05$; ** $p<0.01$; *** $p<0.001$, *** $p<0.0001$. (Control; N= 16, *n*= 20; CD; N=12, *n*= 18; Mann-Whitney, t-test, linear regression, ANOVA).

3.2.6. Non-detectable levels

The proportion of samples which expressed MMP's or TIMP's was also taken into consideration as levels of MMP-1 and MMP-3 are have low expression levels under healthy conditions. For MMP-1 and MMP-3, there were significant differences ($p<0.05$, Fisher's exact test) between the proportion of biopsies in the control patients that did not produce detectable levels of MMP transcript, when compared with CD biopsies (figure 34). The occurrence of non-detectable transcripts was presented in 33% of control biopsies in MMP-1 measurements, and 46.7% of control biopsies in MMP-3 measurements, compared with 0% in CD in both instances. There remained 100% expression levels in all other MMP and TIMP-1 samples.

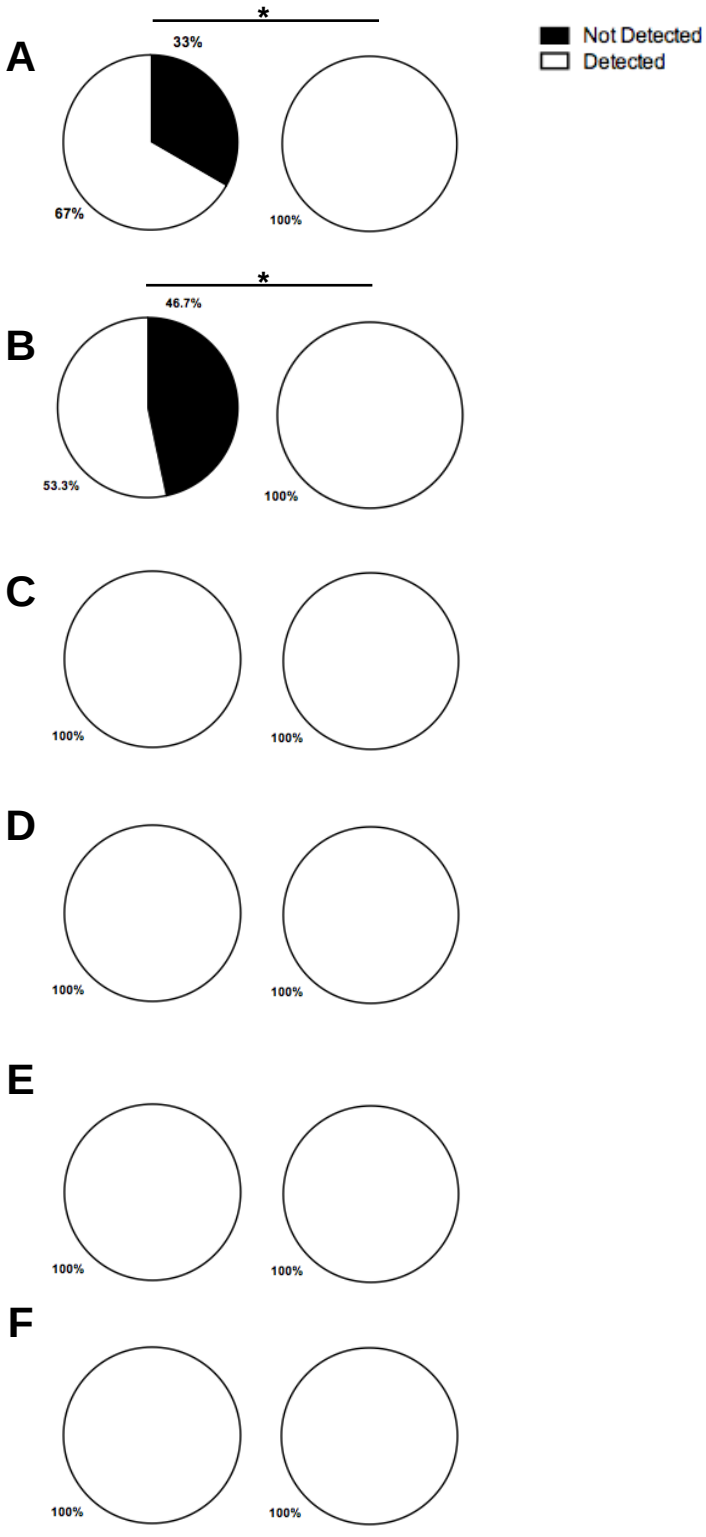

*
A
33%
67%
100%
Not Detected
Detected
*
B
46.7%
53.3%
100%
C
100%
100%
D
100%
100%
E
100%
100%
F
100%
100%

Figure 25. The proportion of biopsies with detectable levels of MMP mRNA.

The mRNA detectable levels in CD biopsies (right) of **(A)** MMP-1, **(B)** MMP-3, **(C)** MMP-9, **(D)** MMP-12, **(E)** MMP-19 and **(F)** TIMP-1 compared with control patients (left). A significant change in the detectable levels was observed between the control patients and CD patients for MMP-1 ($p<0.05$) and MMP-3 ($p<0.05$) levels where the CD patients had more detectable levels of these MMP's. All CD biopsies reported mRNA for all MMP's and TIMP-1 (Control; N= 16, *n*= 20; CD; N=12, *n*= 18; t-test).

3.2.7. TIMP-1

With the TIMP-1 expression, the endogenous inhibitor of MMP's, the median expression was shown to be significantly raised when compared with control samples ($9.3x10^{-4}$ ($6.3x10^{-4}$ – $2.7x10^{-2}$) vs $4.6x10^{-3}$ ($3.3x10^{-3}$ – $1.2x10^{-2}$), respectively, $p<0.05$), and as with the MMP's, drug-naive patients showed a mean raised expression of TIMP-1 mRNA compared with drug-treated (0.020 ± 0.006 vs 0.013 ± 0.005). Although a natural inhibitor of MMP's, there did not appear to be a correlation between TIMP-1 expression and afferent firing in the CD samples ($p=0.27$, $r^2=0.02$).

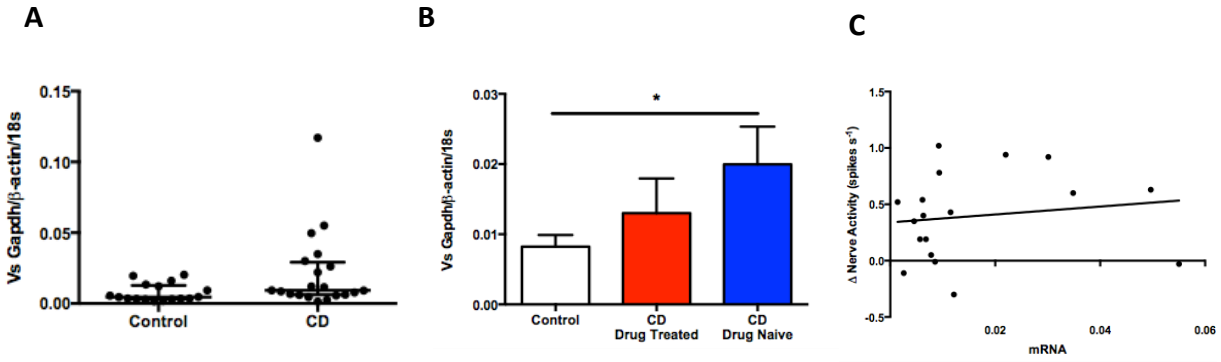


Figure 26. The transcript levels of TIMP-1 in Crohn's disease biopsies.

(A). The mRNA levels in CD biopsies compared with control patients show a significant increase in TIMP-1. **(B)**. This increase was seen to be much higher in patients who were drug-naive compared with patients already receiving treatments and significantly greater than control patients. **(C)**. No correlation was found between TIMP-1 transcript levels and afferent firing. Bars represent mean ± S.E.M. Value where * $p<0.05$.

Bars in expression data represent median (IQR) whereas bar in drug treatment data represent mean ± S.E.M values where * p<0.05; ** p<0.01; *** p<0.001, *** p<0.0001. (Control; N= 16, *n*= 20; CD; N=12, *n*= 20; Mann-Whitney, t-test, linear regression, ANOVA).

3.2.8. The exogenous effects of MMP-12

From the MMP transcript data the correlation between MMP-12 and afferent firing was statistically relevant suggesting that MMP-12 influences afferent firing. To understand if this was a result of a direct action of MMP-12, for example cleaving a GPCR to elicit an action potential, it was applied exogenously to the receptive fields. More indirect actions such as modulating membrane excitability, were also tested by assessing its effects in combination with an inflammatory soup (I.S).

To assess the effects of exogenously applied MMP-12 to colonic afferents, MMP-12 catalytic domain and full length protein were used (figure 26). Compared with the vehicle (0.00 (-0.03 – 0.03) spikes/s^{-1}), the catalytic domain produced significantly greater afferent activation ($p<0.001$) in 3/10 applications (0.17 (0.16 – 0.18) spikes/s^{-1}). The MMP-12 full length protein exhibited a response of similar magnitude to the catalytic domain (0.18 (0.17-0.22) spikes/s^{-1}) which was significant compared with vehicle ($p<0.01$) in 3/8 applications. Mesenteric receptive fields were incubated with 50nM MMP-12 catalytic domain (*n*=9, N=5) but did not result in afferent firing.

To understand the effects of MMP-12 on mechanical responses, a 7 minute incubation of the MMP-12 catalytic domain was applied to serosal layer and mesenteric receptive fields (figure 27). The VFh probing was applied pre- and post-incubation and it was observed that there was no effect on serosal layer mechano-sensitivity with 6.95 (6.10-8.15) spikes/s^{-1} (pre-), vs 7.14 (6.81-7.71) spikes/s^{-1} (post-) (*n*=7, N=5). When MMP-12 was applied to mesenteric receptive fields a significant increase ($p<0.01$) in the probed response was observed (14.22 (13.25-17.36) spikes/s^{-1}, pre- ; 19.56 (17.69-23.34) spikes/s^{-1}, post-, *n*=9, N=6). The effect of MMP-12 on mechanical sensitivity was reversed by using an MMP-12-specific inhibitor, 2μM MMP408, where the baseline probing response was 22.9 ± 4.40 spikes/s^{-1}, and 20.9 ± 4.02 spikes/s^{-1} post-MMP-12 with inhibitor (*n*=3, N=3), which represented a non-significant difference (figure 28).

The indirect effects of MMP-12 were also observed by applying a short incubation of MMP-12 catalytic domain before an inflammatory soup (I.S). The typical response of two incubations of I.S is shown in figure 29 where a reduction in afferent firing can be seen from the second incubation (-36.82 ± 14.93%). When pre-treatment with MMP-12 catalytic domain occurs, this second incubation demonstrated in increase in the afferent firing to 15.47 ± 10.39%, a statistically significant increase ($p<0.05$).

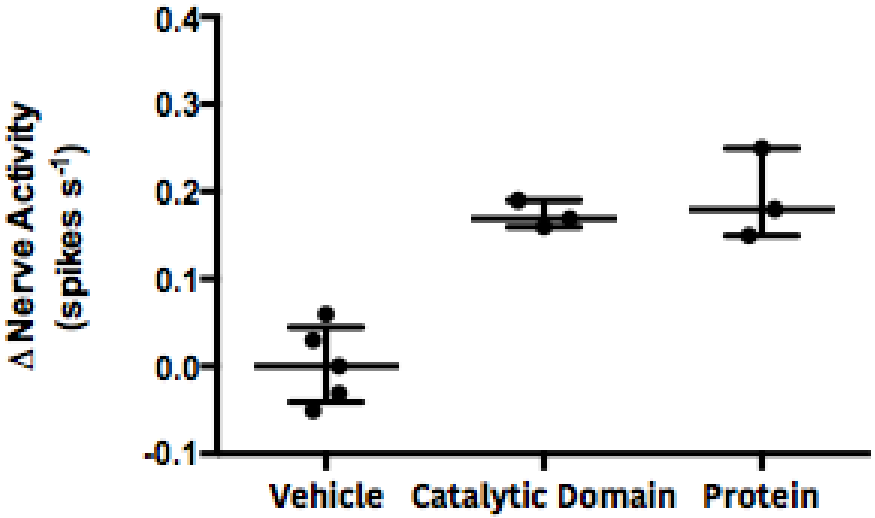


Figure 27. The effect of MMP-12 on serosal layer afferents in C57BL/6 mice.

Vehicle effect was minimal while both the catalytic domain and full-length MMP-12 protein produced significantly greater levels of activation from serosal layer afferents. (N= 5 , *n*= 5; t-test).

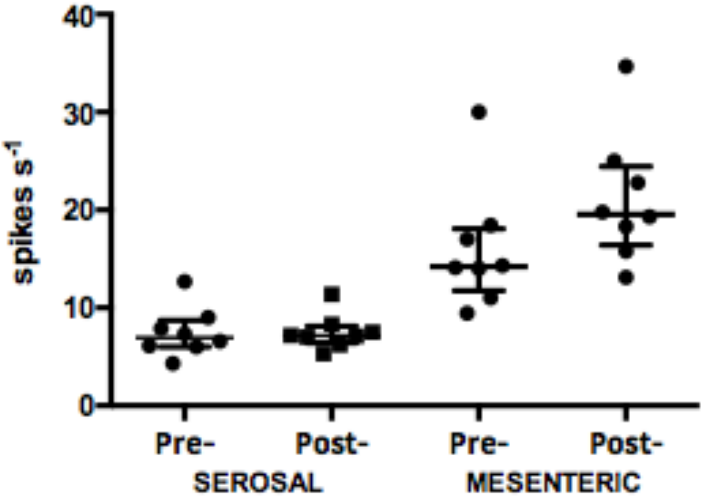


Figure 28. The effect of MMP-12 on mechanical responses to probing.

Serosal layer receptive fields did not show any change in mechanical responsiveness to 1 g VFh after a 7 minute incubation with 50nM MMP-12 catalytic domain. Mesenteric layer receptive fields did show a

significant increase in mechano-sensitivity when a 7 minute incubation of 50nM MMP-12 catalytic domain was applied. (Serosal; N= 5 , *n*=7 ; mesenteric; N= 6 , *n*=9; t-test).

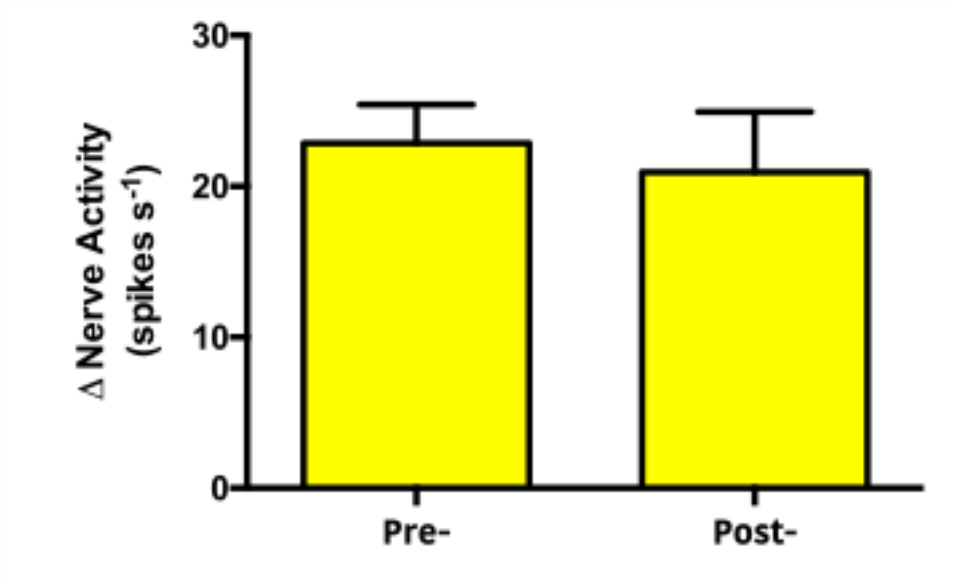
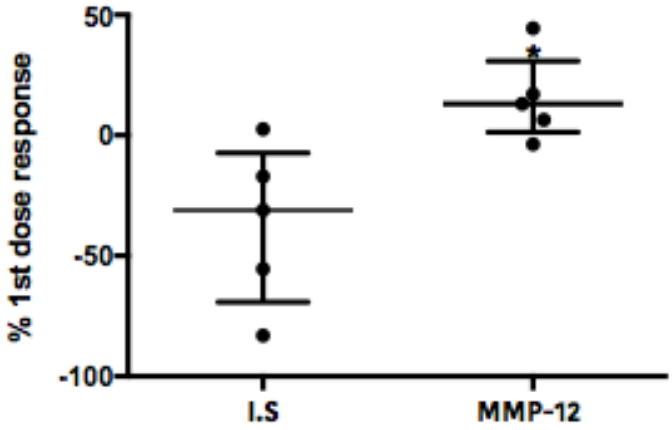


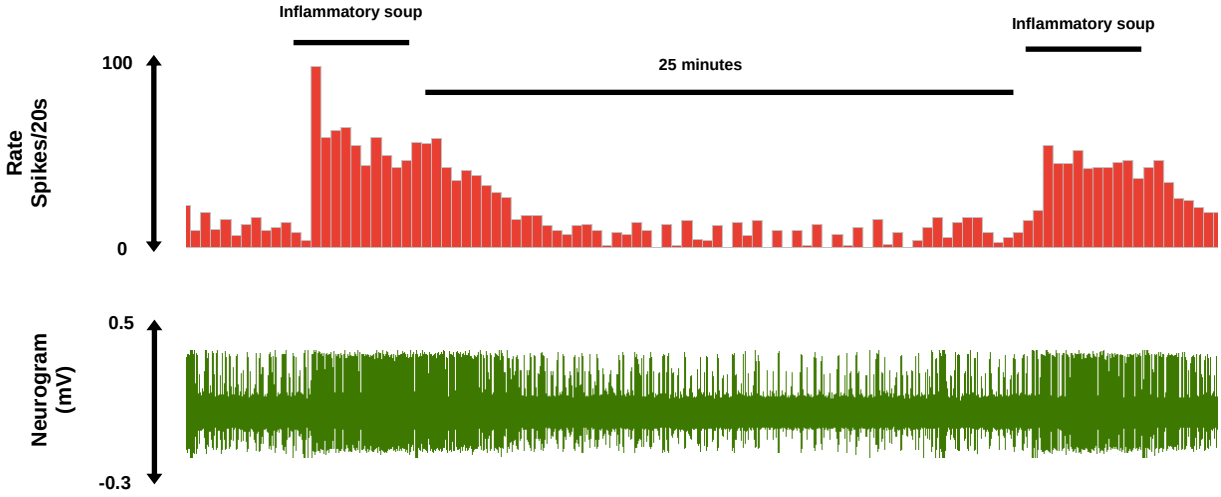
Figure 29. The effect of an MMP-12 inhibitor on mesenteric mechanical responses.

Mesenteric layer receptive fields were tested pre- and post- 50nM MMP-12 catalytic domain incubation in the presence of 2µM MMP408 which abolished the sensitisation effects of MMP-12 on mechanical probing. Bars represent mean ± S.E.M. (N= 3 , *n*= 3 ; t test).

A


50
0
-50
-100
% 1st dose response
I.S
MMP-12

B


Inflammatory soup
Inflammatory soup
25 minutes
100
0
Rate
Spikes/20s
0.5
-0.3
Neurogram
(mV)

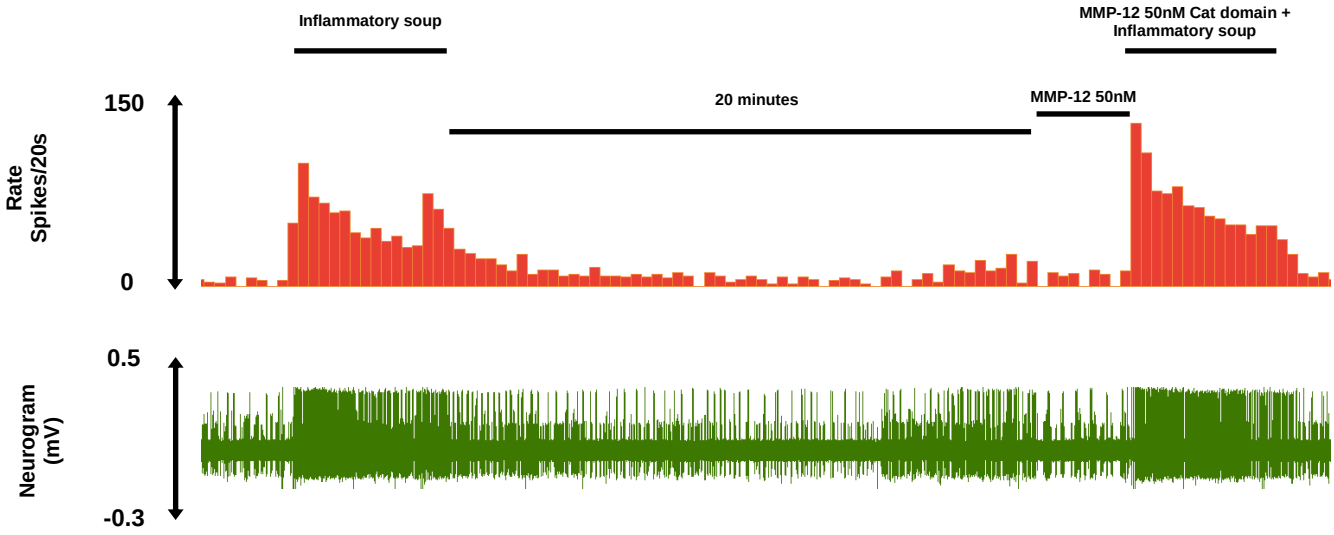

Inflammatory soup
MMP-12 50nM Cat domain +
Inflammatory soup
20 minutes
MMP-12 50nM
150
0
Rate
Spikes/20s
0.5
-0.3
Neurogram
(mV)

Figure 30. The effect of MMP-12 pre-incubation with an inflammatory soup.

(A). The inflammatory soup produced a reduced level of activation when given for a second time 25 minutes after the first dose. This desensitisation of the second response was abolished when the receptive field was pre-treated with 50nM MMP-12 catalytic domain. The response of the inflammatory soup was increased due to the presence of MMP-12. **(B).** Responses shown are represented as a percentage of the first effect on the inflammatory soup which showed a significant difference. I.S; inflammatory soup. (N= 5, *n*= 5; t test).

3.2.9. Preliminary data for the exogenous application of MMP-12 on human afferents

With the effects of MMP-12 established on mouse colonic afferents, next MMP-12 was applied to serosal receptive fields from resected human descending colon. The resulting application (*n*=3, N=3) led to an increase in firing of 0.28 ± 0.23 spikes/s^{-1} with the vehicle (*n*=1, N=1) causing little change in firing (0.06 spikes/s^{-1}). The mechanical responses were also tested pre- and post- MMP-12 application (*n*=2, N=2) and were found to increase post-MMP-12 application (from 6.0 spikes/s^{-1} to 8.89 spikes/s^{-1}, and 7.67 spikes/s^{-1} to 10.44 spikes/s^{-1}).

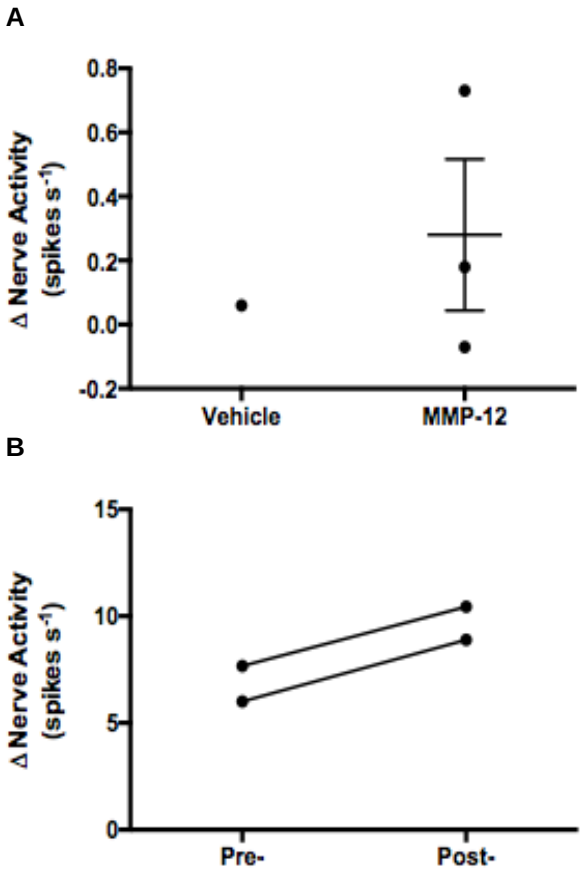


Figure 31. Human afferent responses to MMP-12 application

(A). The vehicle response compared to the response of MMP-12 on human serosal afferents where in 2/3 applications, a greater level of afferent firing was observed. MMP-12 shows mean ± S.E.M (N=3, *n*=3) **(B).** In 2 serosal units tested with VFh pre- and post-MMP-12 application, the afferent demonstrated increased excitability to mechanical stimuli. (N= 1 , *n*= 1)

3.2.10. The effects of MMP-9 are similar to MMP-12 on mouse colonic afferents

MMP-9 is released from macrophages in much a similar manner as MMP-12 release. Combined with earlier observations in which MMP-9 transcript levels were increased in biopsy tissue, MMP-9 was exogenously applied to mouse serosal layer afferents following the same protocol as MMP-12 application. It was observed that a strong level of afferent firing resulted from the application of MMP-9 (*n*=5, N=5) compared with vehicle (0.29 (0.21-0.30) spikes/s^{-1} vs 0.00 (-0.03-0.03) spikes/s^{-1}; $p<0.001$). The same application was tested on mesenteric receptive fields (25nM, *n*=4, N=4; 75nM, *n*=2, N=2) but no response observed (0.00 spikes/s^{-1}). Mechanical responses were also tested pre- and post-MMP-9 application with 1g VFh in both serosal (*n*=4, N=4) and mesenteric (*n*=4, N=4) receptive fields. There was a small but statistically non-significant reduction in mechanical response in serosal units tested (pre-, 8.78 (7.67-10.0) spikes/s^{-1}; post-, 7.59 (6.90-8.25) spikes/s^{-1}). In mesenteric afferents, there was a non-significant increase in mechanical response post-application of MMP-9 (pre-, 19.35 (18.14-21.00) spikes/s^{-1}; post-, 23.54 (20.22-26.20) spikes/s^{-1}).

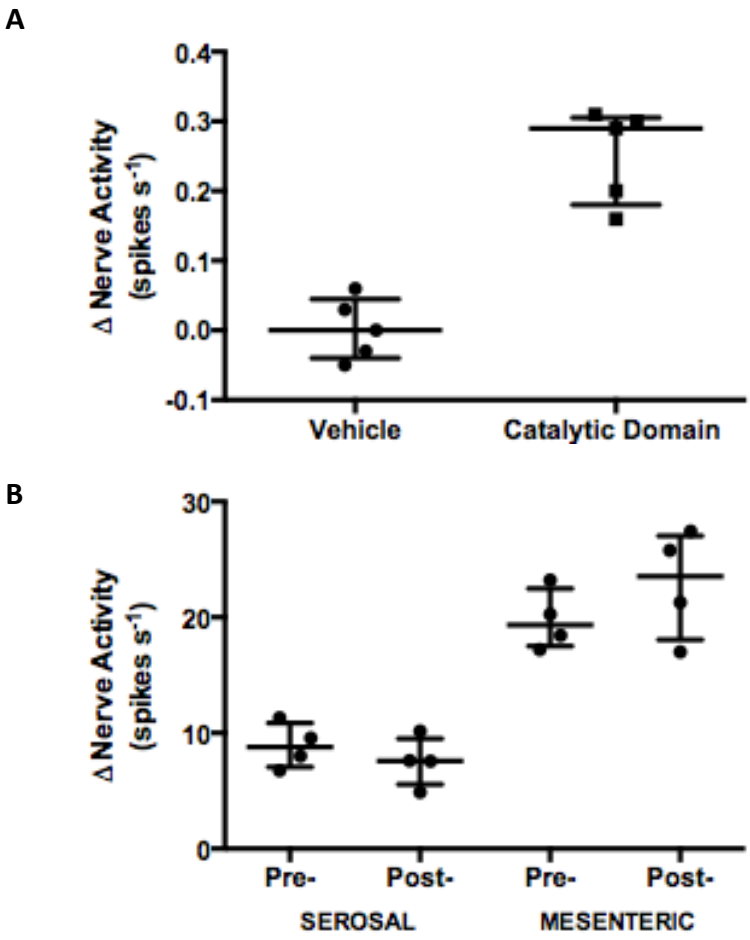


Figure 32. The response of mouse colonic afferents to MMP-9

(A). The addition of MMP-9 catalytic domain (25nM) produced a significant increase in afferent firing compared with vehicle ($p<0.001$) (vehicle; N=5, *n*=5; MMP-9; N=5, *n*=5). **(B).** There was a small but non-significant increase in mechanical response in mesenteric units following the application of MMP-9. Bars represent mean ± S.E.M. (Serosal; N=4, *n*=4; mesenteric; N=7, *n*=7) (Mann-Whitney).

3.2.11. Preliminary data shows the effects of MMP-9 with an inflammatory soup

As we have demonstrated that MMP-12 can influence the response of the afferent to chemical stimuli from an inflammatory soup (I.S), next it was important to understand whether MMP-9 had similar characteristics. Following the same protocol, MMP-9 was added prior to the second incubation of the I.S (*n*=2, N=2) and it was observed that in 40% of applications, the I.S response was greatly increased (106.1% increase from

control dose). Control dose (2nd incubation of I.S) produced a reduced response (*n*=5, N=5) in afferent firing (-36.2 ± 14.9%; as a percentage of dose 1).

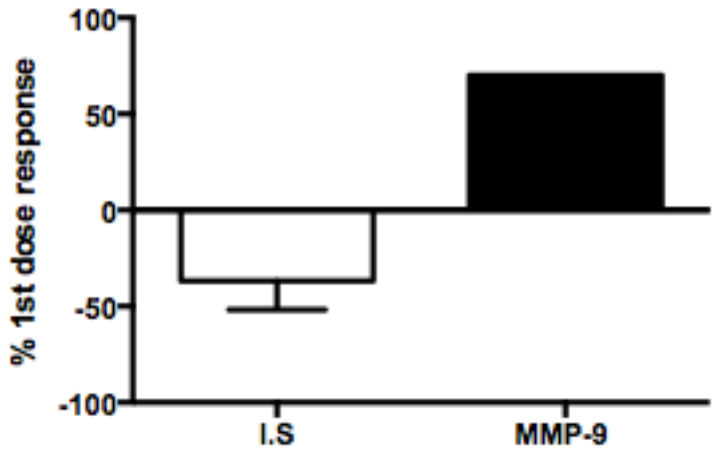


Figure 33. The effect of MMP-9 on an application of inflammatory soup

Pre-incubation of MMP-9 catalytic domain (25nM) prior to a secondary dose of inflammatory soup (I.S) eliminates the desensitising effect resulting in an increase in a afferent response. Bars represent mean ± S.E.M. (N= 2 , *n*= 2).

3.2.12. Macrophage cell markers

To identify macrophage infiltration in the biopsies, cell markers were assessed by qPCR (figure 33). In the CD biopsies there was a significant increase in the median expression of TLR_4 compared with controls ($1.9x10^{-3}$ ($1.7x10^{-3}$ – $3.2x10^{-3}$) vs $1.2x10^{-3}$ ($8.9x10^{-4}$ – $1.4x10^{-3}$), $p<0.01$). TLR_4 drug-treated group measured a mean response of; 0.0021 ± 0.0003 ($p<0.05$), drug-naive; 0.0028 ± 0.0008, ($p<0.05$), vs control 0.0010 ± 0.0003). The expression of TLR_4 was compared with the afferent firing and was not statistically significant ($p=0.43$, $r^2=0.03$). With the macrophage makers CD14 and CD16, there was an increased median expression of both in CD compared with controls (CD14; $8.1x10^{-3}$ ($4.8x10^{-3}$ – $1.0x10^{-2}$) vs $5.7x10^{-3}$ ($3.9x10^{-3}$ – $7.9x10^{-3}$), respectively; CD16 $4.2x10^{-5}$ ($3.4x10^{-5}$ – $8.4x10^{-5}$) vs $3.0x10^{-5}$ ($2.7x10^{-5}$ – $3.3x10^{-5}$). There was a non-significant increase in the expression of CD14 in the drug-naive group and a noticeable mean increase in the drug-treated group when compared with afferent firing ($p=0.27$, $r^2=0.04$). The CD16 was increased in drug-naive

patient biopsies compared with drug-treated and controls, although many samples did not show expression above the lower limit of detection and so comparisons were made difficult (CD14 drug-treated; 0.0077 ± 0.0014, drug-naive; 0.0095 ± 0.0021; CD16 drug-treated; 4.0 x 10-5, *n*=2, N=2; drug-naive; 5.3 x 10-5 ± 2.2 x 10-5, *n*=3, N=3).

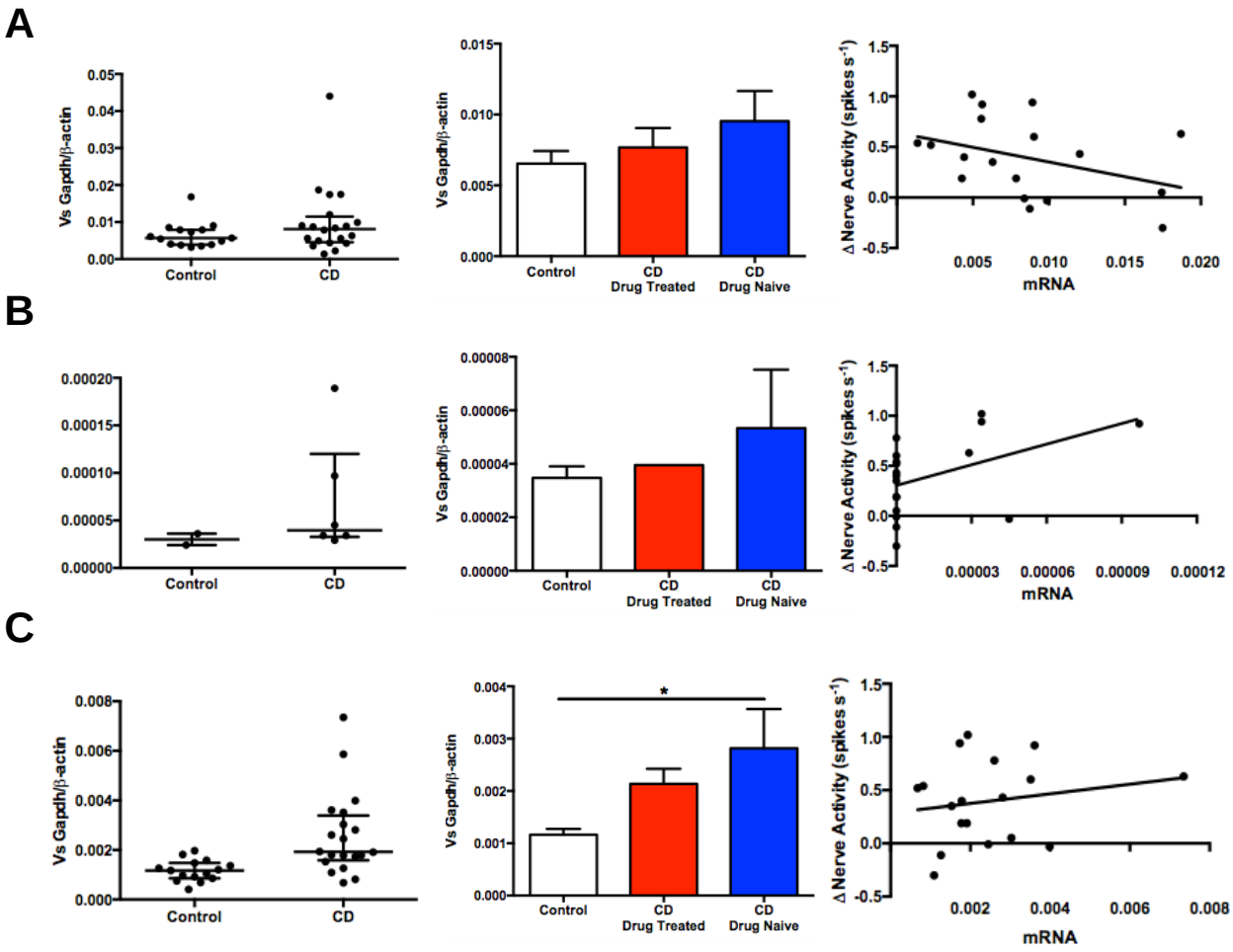


Figure 34. The transcript levels of macrophage markers in Crohn's disease biopsies

(A) The mRNA levels in CD biopsies of CD14 (control; N= 15, *n*=15; CD; N=12, *n*=20), **(B)** CD16 (control; N= 3, *n*=3; CD; N=6, *n*=6), **(C)** TLR_4 compared with control patients (control; N= 15, *n*=15; CD; N=12, *n*=20). A significant increase levels of TLR_4 in CD biopsies compared with controls. This was also observed when patients were stratified into drug-treated and drug-naive, with the CD14 marker significantly increased in drug-naive patients compared with controls. A significant correlation was observed between the transcript levels of CD14 and afferent nerve activity ($p<0.05$). Bars in expression represent median (IQR). Bars in drug groups represent mean ± S.E.M values where * $p<0.05$, ** $p<0.01$. (Mann-Whitney, t-test, linear regression, ANOVA).

3.2.13. Macrophage release of MMP's

Macrophages have been documented as a major source of mmp-9 and mmp-12 and so the macrophage marker tlr_4 was correlated with both mmp's to understand if a linear relationship existed in the samples used in this study. Mmp-9 correlation showed r^2=0.47, p<0.0001; mmp-12 correlation showed r^2=0.38, p<0.003.

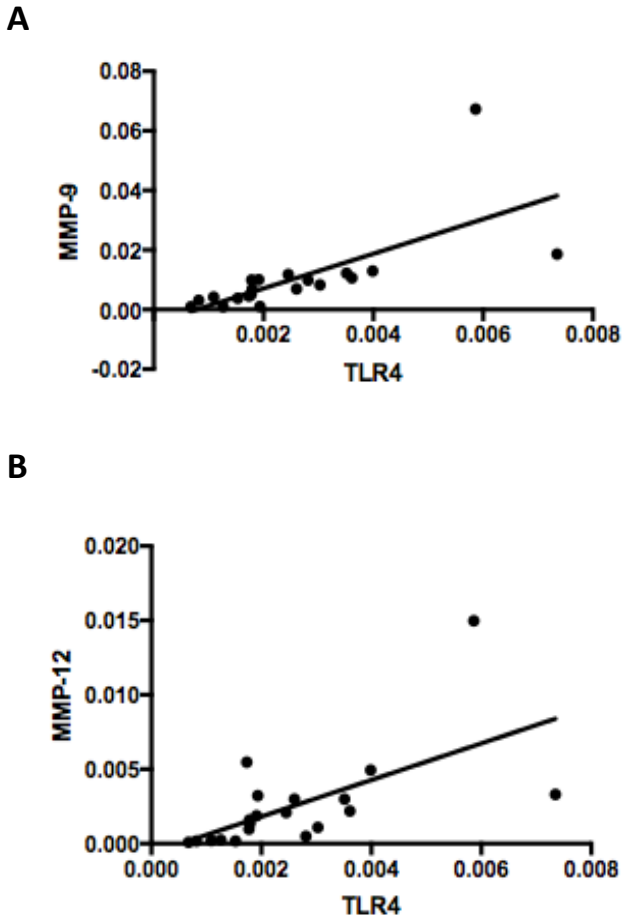


Figure 35. Macrophages as a potential source of MMP's

(A). Shows the positive linear relationship between the transcript expression for the macrophage marker TLR_4 and MMP-9. **(B).** Shows the positive linear relationship between the transcript expression for the macrophage marker TLR_4 and MMP-9. TLR; Toll-like receptor; MMP; matrix metalloproteinase. (N= 12, *n*= 20; linear regression, ANOVA).

3.2.14. SUMMARY OF RESULTS

- ❖ Evidence of mucosal inflammatory processes in biopsies
- ❖ Supernatants are capable of robust colonic afferent activation in rodents and humans suggesting a pro-nociceptive gut environment
- ❖ No change in mechanosensitivity from supernatants was observed
- ❖ Drug treatments aimed at immunosuppression do not affect colonic afferent activation
- ❖ MMP-12 levels correlate with afferent firing and exogenously applied MMP-9 or MMP-12 cause afferent firing in both rodents and humans and increases the mechanical sensitivity of mesenteric afferents in rodents
- ❖ MMP-9 and MMP-12 also enhance the ability of pro-nociceptive mediators to elicit afferent firing
- ❖ Macrophages appear to be a likely source of both MMP-9 and MMP-12

3.3. DISCUSSION

3.3.1. Overview

Crohn's disease is a chronic inflammatory condition which can affect all of the GI tract but often occurs in the ileocecal region and colon. One of the most debilitating symptoms is severe abdominal pain (table 1, 4)). As it is an immunological disorder current thinking is that pain is driven by inflammatory cytokines such as IL-1β and IL-6, and classical mediators such as 5-HT and histamine, and proteases, which have been shown to be capable of eliciting strong activation of colonic afferents (Hughes, 2013; Zhao, 2015; Uçeyler, 2007; Cenac, 2007; Jimbo, 2014, Matusiewicz, 2009; Gordon, 2008; Atreya, 2000; Dionne, 1998; Radiate, 2005). These mediators have also been shown to sensitise afferent endings often through phosphorylation of ion channel such as the TRP channels and voltage-gated sodium channels (Jin & Gereau, 2006; Zhang, 2005; Caterina, 2000; Amaya, 2006). Changes in mechano-sensitivity, essentially resulting in extreme pain for patients when eating and digesting food, has also been linked to inflammatory cytokines such as TNF-α (Hughes, 2013). This study focused on attempting to understand activation and acute sensitisation of colonic afferents using CD biopsies. Therefore, pain-related mediators such as classical neuromodulators (PGE_2), proteases (tryptase, elastase, MMP's), cytokines (IL-1β, TNF-α, IL-6) were assessed.

3.3.2. Afferent response to supernatants

The effect of the biopsy supernatant was tested on mouse serosal layer receptive fields (figure 12). The vehicle used during the biopsy incubation was tested on the afferent and no effect was observed to ascertain that any effect from disease or control biopsies must be due to the disease pathology or tissue damage. The control samples elicited a small and consistent activation from colonic afferents in agreement with local damage to the tissue from the biopsy procedure, already discussed in chapter 2. The CD supernatants produced a robust activation significantly greater than the controls with the peak levels of activation nearly 10-fold greater, suggesting that the colonic environment in CD is not just pro-inflammatory but also centered around a pro-nociceptive state meaning that regardless of changes in peripheral or central nerves the local environment is likely more 'painful' than a normal gut environment. This is consistent with current literature which demonstrates elevated pro-inflammatory and pro-nociceptive mediators in colonic mucosal tissue in CD (Atreya, 2000; Dionne, 1998; Vasilyeva, 2016; Mahida, 1991).

To understand if the mouse colonic afferent study translates to human nociceptors a small number of CD supernatants were also tested on human serosal afferents and it was found that the supernatants were capable of producing high levels of activation when incubated on the receptive field (figure 13). This means that CD biopsies have the capacity to release mediators capable of stimulating human colonic nociceptors, potentially translating to pain in patients. The human tissue used ofr afferent recordings was resected from adult CD patients with extensive chronic inflammation. The ability of the supernatant to activate human afferents that have been exposed to an inflamed endogenous environment demonstrates how robustly inflammatory mediators can elicit nociception. Encouragingly, this approach validates the biopsy supernatant model used in naive afferents in mice used throughout this study, as there appears to be a direct relationship between supernatant responses in uninflamed mouse colon and human colon, presumably, hypersensitive to inflammatory mediators.

3.3.3. Patient pain scores and afferent response

When initially consented, patients were asked to score their perceived levels of abdominal pain based on a 0-3 VAS range. Two time periods were considered, the pain on the day of biopsy removal, and the maximum pain level faced in any day from the previous month. When the afferent firing rates from each patient was coupled to either of their pain scores a trend towards greater afferent activation in higher pain scoring patients became apparent (figure 21). This suggests that the supernatant response on the afferent, although only a measure of afferent activation and nociception, may have a potential role in translating to patient pain measures. This trend in elevated afferent responses in patients who reported the greatest pain scores was not statistically significant although it does suggest that the afferent recording technique may be suitable for a diagnostic tool, to decipher pain levels in young children who are unable to communicate it, or patients who have difficulties comprehending symptoms to guide the clinician. Potentially, it may also have a use to deliver a prognosis of visceral pain. Although it is a promising preliminary finding, this data however, should be interpreted with some level of caution. Firstly, the 0-3 VAS rating may not offer a range with enough variation for patients to distinguish between pain levels, which could result in a high degree of error and a larger cohort of patients would undoubtedly be needed meaning that a follow-up study with a potential to

record pain in real-time, thereby not relying on historical pain scoring, in a larger patient-led study would be beneficial to understand.

As mechanical sensitivity is a key part of visceral pain in CD, the VFh probed responses of the receptive fields were assessed before and after the incubation of CD biopsy supernatants (figure 14). It was observed in this study that the CD supernatants did not cause any significant changes to the mechano-sensitivity towards probed responses. Contrary to current literature which demonstrates how supernatants from inflamed tissue can lead to mechanical sensitisation through ion channel adaption such as voltage-gated sodium channels which have been shown to be coupled to $TRPV_1$, $TRPA_1$, and $TRPM_8$, channels (Schmidt, 1995; Harrington, 2011; Ibeakanma, 2009). Changes in mechanical sensitivity of sensory neurons has been demonstrated and may explain why patients within the CD population did not show mechanical changes after supernatant incubation as many patient were undergoing anti-TNFα treatments which likely effect the concentration of TNFα to bind to $TNFR_1$ which has been shown to be coupled with $TRPA_1$, a transducer shown to be involved in mechanical sensitivity. However, supernatants from patients in this study who were drug-naive did not show changes in mechanical sensitivity although there is potential in this study for a sub-maximal weight of VFh probe to have been used to observe subtle changes in neuronal threshold changes.

3.3.4. Drug treatment grouping

As this study consented patients presenting with an initial diagnosis of CD it gave the opportunity to stratify patients into those who were not yet receiving treatments (drug-naive) and those with a history of treatments (drug-treated) (table 2, 3). When analysing supernatant levels of the neutrophil chemoattractant IL-8, used here as a surrogate marker for a local inflammatory response, a significantly reduced IL-8 level was observed in samples from drug-treated patients (figure 22). which suggests that the current drug-therapy, used for targeted immunosuppression, is efficacious. However, when afferent firing rates were compared between the two treatment groups, no difference was observed suggesting that immunosuppression does not necessarily result in a reduced nociceptive environment in the gut, and this is evident from patients in this study who report abdominal pain regardless of treatment by immunosuppressants. Therefore it is hypothesised in this study that mediators which are not targeted by current immunosuppressive therapies

are responsible for abdominal pain.

3.3.5. Cytokine involvement in afferent response

This next stage of the study looked to identify mediators that were responsible for afferent activation. Due to compelling evidence of pro-inflammatory cytokine involvement in the pathology of IBD and direct afferent activation, IL-8, IL-1β, IL-6, and TNFα, levels were assessed in relation with afferent firing (figure 21). Several laboratories have described how inflammatory cytokines elicit changes in sensory afferent firing (Burnstock, 1996; Obreja, 2002; Rush & Waxman, 2004; Brenn, 2007; Richter, 2012; Hughes, 2013). Despite cytokines being shown to modulate afferent activity either directly through specific rececptors expressed on sensory neurons such as the IL-1R, or indirectly via activation of further inflammatory cytokines such as IL-8. No correlation was observed between cytokine transcripts and afferent firing, suggesting that in this study, these cytokines are not responsible for modulating afferent activity (figure 22, table 8). This difference could be explained by appreciating that high transcriptional levels may not directly result in high protein levels in the supernatants due to the relatively short biopsy incubation time. Considering this, it remains likely that there may be other mediators that have a more prominent role in the supernatants.

3.3.6. MMP involvement in afferent response

Other prospective mediators responsible for abdominal pain in CD included matrix metalloproteinases (MMP's). MMP's are a family of proteases that degrade the extracellular matrix digesting denatured collagens, but have been the focus of interest as an inflammatory mediator in IBD, and have also been linked with various types of pain (Moore, 2011; Kofla-Dłuback, 2014**;** Jimbo, 2014**;** Matusiewicz, 2014; Yamamoto, 2003; Kawasaki, 2008). Based on current literature, this study focused on 5 MMP's (MMP-1, MMP-3, MMP-9, MMP-12, MMP-19) where elevated expression of all MMP's was observed (figure 24, 25). Although only transcript levels were measured, a previous body of work indicates that mRNA levels of the MMP family accurately correspond to protein levels (von Lampe, 2000; Moore, 2011; Wang, 2009; Monteleone, 2006). In all instances, MMP levels were greater than control samples regardless of whether the patient was on drug treatment suggesting that MMP's are not targeted by current CD therapy and any potential

dysregulation may go without therapeutic control. In addition to this, the proportion of CD samples where MMP expression was detected was always greater, or equal to that of control samples adding to evidence that MMP's are upregulated during chronic inflammation. From all MMP's studied here, it was observed that MMP-12 levels positively correlated with afferent firing suggesting that this protease may influence afferent activity (figure 23). Considering this, It was observed that the supernatants which did not cause an afferent response had a significantly reduced expression of and MMP-12 and elevated expression correlated with increased afferent firing (figure 24). Therefore it is likely that these MMP's play a prominent role in afferent activation. This is the first time that MMP-12 has been associated with nociception and abdominal pain. Serum MMP-2 and MMP-9 have been shown to have a direct link with arthritic pain and abdominal pain but no studies have shown a direct correlation with any MMP and afferent activation (Jimbo, 2015). This study therefore suggests that MMP-12 could be associated with abdominal pain in CD.

Importantly, the levels of the endogenous MMP inhibitor TIMP-1 were also significantly elevated in CD samples and were not effected by patient drug treatments (figure 25). This balance of effector and inhibitor is a key component of IBD, however, given the strong association between MMP-9 and MMP-12 with afferent firing it is likely that the TIMP-1 levels remain too low to inhibit all MMP activity.

In consideration of the correlation between afferent firing and MMP-12 expression, the next approach was to test whether exogenously applied MMP-12 could stimulate colonic afferents. Application of the MMP-12 protein or its catalytic domain produced a robust activation in 30% of afferents tested suggesting that it has the ability to act directly on the afferent ending to elicit downstream action potentials (figure 27, table 7). Although the mechanisms involved are not understood at this time, the G-protein couple receptor PAR_1 has been implicated in MMP signalling and so it is possible that MMP-12 acts through the PAR receptors whereby coupling to ion channels such as $TRPV_4$ could potentially lead to action potential firing (Li & Tai, 2014). In a similar manner, PAR_2 coupling to $TRPV_1$ on C-fibers has been documented, and observed cleavage sites on voltage-gated sodium channels could contribute to a potential mechanism of direct afferent firing (Gu & Lee, 2010; Remacle, 2015).

To understand any influences on mechanical sensitivity from MMP-12 the receptive fields of serosal and mesenteric afferents were incubated with MMP-12 and VFh responses were measured before and after this incubation. MMP-12 had no effect on serosal afferent mechanosensitivity, however MMP-12 produced a significant increase in mesenteric probed responses, an effect blocked by pre-treatment with the MMP-12 inhibitor (figure 29). As the mesenteric receptive fields may be exposed to MMP's during inflammatory disease it is possible that MMP-12 may be contribute abdominal pain under certain inflammatory conditions. Although the mechanism of action is not known, the acid-sensing ion channels (ASIC) have been shown to be involved in mechanical responses in mice. Several laboratories have demonstrated a change in VFh responses or colonic phasic distensions when blocking the activity of ASIC channels (Jones, 2005; Page, 2005). In addition, patterns of expression also show heterogeneity amongst subtypes of afferents. For example, $ASIC_2$ has shown preferential expression in mesenteric afferents over serosal afferents and $ASIC_2^{-/-}$ mice have shown increased responses to VFh probing (Page, 2005). This expression could account for the differential effects on VFh probing in serosal and mesenteric fibers. Furthermore, a recent *in vivo* study showed a PAR_3-specific ligand that was able to induce mechanical hypersensitivity in rodents, and expression of PAR_3 in human DRG's has been observed (Price, 2016). The mechanical sensitivity of human afferents was also tested using VFh probing after MMP-12 incubation and was found to increase suggesting that local changes on mechanically sensitive peripheral nociceptors can be driven by MMP-12.

To understand the translational potential of these observations human resected tissue from CD patients was obtained and nerve recordings of serosal layer nociceptors were also tested using MMP-12. When the MMP-12 was added to the receptive field (figure 31), a large activation was observed in most afferents demonstrating a meaningful contribution to afferent activation from MMP-12 in both rodent and human afferents. Hence, this observation is an important one as MMP-12 is shown to directly activate nociceptors in an inflamed gut. This model resembles the endogenous CD colonic environment remarkably well, meaning that MMP-12 is likely to act in a similar manner in CD patients, thus presenting MMP-12 as a target protein for future pain targeting studies and that MMP's should be considered not only as pro-inflammatory proteases but also as pro-nociceptive mediators.

Although application of MMP-12 elicited an increase in afferent activity, the magnitude of effect was smaller than that observed by the application of CD supernatants. To understand if this difference was due an additive effect of MMP-12 activity in the presence of other mediators, MMP-12 was added to the afferent in combination with an inflammatory soup (BK, ATP, Histamine, 5-HT, and PGE_2). In control studies two applications of inflammatory soup resulted in a robust activation of colonic afferent with the second application showing desensitisation by comparison with the first (figure 30). However, in the presence of MMP-12 a significant increase in afferent activation was observed following a second application of inflammatory soup. Therefore, although when given alone MMP-12 only stimulates 30% of afferents (figure 27), in combination with other mediators MMP-12 enhances afferent activity in a broader range of fibers. The mechanism of MMP-12-mediated afferent activation is unclear. MMP-12 has been shown to induce ATP release from epithelial cells through a PAR_2/$ASIC_3$/$TRPV_1$ mechanism and it is possible that ATP or its metabolites UTP and ADP contribute to the effect of MMP-12 on colonic afferents (Wu, 2015; Gu & Lee, 2010; Zang, 2015; Hockley, 2016). This ATP release may be at suboptimal concentrations for a direct activation and ATP has been widely reported as in inflammatory mediator involved in peripheral sensitisation (Burnstock, 1996; Rong & Burnstock, 2004; Shinoda, 2009; Hockley, 2016). PAR_2 activation of C-fibers can also lead to increased MMP-12 production (Zang, 2015). As inflammatory mediators used in the I.S have been shown to sensitise sensory neurons and lead to changes in neuronal excitability through GPCR and ligand-gated ion channels leading to increased intracellular calcium and activation of voltage-gated sodium channels, this could pragmatically be a mechanism which primes the afferent to MMP-12 cleavage. Interestingly, the proportion of afferents responding to MMP-12 in this study is similar (30%) to the proportion of mouse colonic afferents that express the ATP receptor P_2X_3 (Robinson, 2004). Currently, this study is unable to explain the precise mechanisms of the enhancement of the I.S activity and MMP-12 may possibly act through multiple direct or indirect signalling pathways to achieve an increase in afferent firing.

3.3.7. Afferent response to MMP-9

As macrophages release both MMP-9 and MMP-12, and elevated expressions of MMP-9 was observed within the biopsy samples, MMP-9 was also assessed for pro-nociceptive properties. MMP-9 showed a direct effect on the serosal afferent ending by eliciting a robust activation in 50% of the units tested suggesting that it may act directly on afferent endings. Preliminary data on the effects of MMP-9 on mechanical responses shows a similar patter to MMP-12 whereby the mesenteric afferents may be more effected. However, further investigation into this effect is warranted before conclusive evidence for its effects on mechanical sensitivity are understood.

MMP-9 was also added to the receptive field of a human colonic afferent where a robust increase in afferent firing was observed (figure 32). Although only preliminary in its findings, it is an important observation that supports the translation of MMP-9 nociceptor activation into potential pain mechanisms in patients.

In a similar manner to MMP-12, the MMP-9 was also tested with the addition of the I.S. When the serosal afferent receptive field was pre-incubated with MMP-9 prior to the second dose of I.S, an amplified level of firing resulted from the I.S suggesting that it has similar modulating activity on peripheral afferents as MMP-12 (figure 33). This is an important observation as it is tenable that a synergistic relationship exists with the release of multiple MMP's and inflammatory mediators.

Although there is no clear mechanism of action for the effects of MMP-9 on afferent activation, an *in silico* study found an MMP-9 cleavage site on $Nav_{1.7}$, a voltage-gated sodium channel known to be involved in pain (Remacle, 2015). Cleavage of this sodium channel by MMP-9 could plausibly lead to a hyper-excitable afferent which when accompanied by mediators within the I.S results in enhanced afferent firing. As MMP-9 is released from macrophages in a similar manner to MMP-12, it is possible that they share a similar mechanism of action. In line with this, MMP-9 may also lead to the activation of the PAR_2/$ASIC_3$/$TRPV_1$ pathway that may play a role in sensitisation and activation of sensory afferents.

Within the inflamed gut, it is likely that the increased inflammatory cell recruitment results in the release of multiple MMP's. In light of this, an inflammatory environment which results in elevated levels of MMP's likely contributes to altering the peripheral neuro-immune interactions towards a pro-nociceptive environment. By understanding these mechanisms further and targeting potential dysregulation of MMP's,

in particular MMP-9 and MMP-12 within the inflamed gut, future pain treatments may benefit.

3.3.8. Macrophage infiltration and release of MMP's

Further analysis of the biopsy mRNA identified immune cell markers to understand the contribution of different cell types within the mucosa. As MMP's were identified as potential mediators in nociception, monocyte and macrophage expression was evaluated. This study observed modest increases in the expression of monocyte markers CD14 and CD16, however, the proportion of detectable levels of CD14 was far greater than CD16 (figure 34, 35). This is consistent with the understanding that approximately 90% of human monocytes are $CD14^{+}/CD16^{-}$ which differentiate into classical pro-inflammatory macrophages. Although this study observed greater overall expression of $CD14^{+}$ monocytes, there is a current debate within literature as to the precise expression patterns of monocyte subtypes with CD, and how they compare with UC (Koch, 2010; Thiesen, 2014; Kühl, 2015).

Our results did show an elevated expression of the macrophage marker TLR_4 (figure 34).Furthermore, this expression was coupled to MMP-9 and MMP-12 expression suggesting that M1 macrophages, are responsible for the release of both MMP's, however, further investigation would be needed to confirm this conclusion. Interestingly, this finding may have broader implications as both macrophages and MMP's have been associated with neuropathic pain suggesting that an approach limiting macrophage release of MMP's may be a valuable future therapeutic target in multiple diseases where severe pain is a clinical symptom (Kobayashi, 2015).

Furthermore, greater investigation into the endogenous of MMP's in inflammatory pain would be of great benefit. For example, the role of MMP's in neurogenic inflammation has not yet been fully explored. PAR_2-positive C-fibers have been shown to release SP thereby encouraging the release of MMP-12 from nearby macrophages via NK-1 receptor activation (Zhang, 2016). CGRP has been shown to contribute to neuro-immune mechanisms and specifically mast cell release of inflammatory mediators (Assas, 2014). MMP-12 and mast cell mediators such as tryptase, TNFα, and ATP, could act in a positive feedback loop on C-fibers promoting a sensitised peripheral environment and contributing to chronic pain.

3.3.9. Conclusion

In conclusion, supernatants derived from CD patient colonic biopsies elicit afferent firing in both mouse and human nociceptors. Comparison of afferent firing with biopsy transcript expression revealed a significant correlation between afferent activity and MMP-12 expression. Exogenous application of MMP-9 and MMP-12 caused a direct activation of serosal afferents in rodents and humans. Additionally, MMP-9 and MMP-12 sensitised responses to mechanical stimuli and an inflammatory soup suggesting a likely contribution to nociception and visceral pain in patients with IBD.

CHAPTER 4: ULCERATIVE COLITIS

4.1. RESULTS

Supernatants generated from UC biopsies cause hyper-excitability in mouse nociceptors through the actions of TNFα and this mechanical allodynia in C-fibers has been suggested to be mediated through TNF_{R1} activation leading to modulation of $Nav_{1.8}$ and K_V channels.

TNFα has been also been shown to act directly on mouse visceral afferent endings to induce mechanical hyperalgesia in keeping with previous observations of TNFα causing mechanical allodynia in rat DRG's.

In UC, IL-17 has also been shown to lead to increases in inflammatory proteases such as MMP-1 and MMP-9 at both the protein and mRNA level and as MMP-9 has previously shown involvement in neuropathic pain conditions it remains likely that both mediators may share some properties in visceral pain. TIMP-1, the endogenous inhibitor of MMP's is an important protein to consider when understanding the influence of MMP's within the endogenous environment. Four subtypes are known to exist, although so far only the actions of TIMP-1 and TIMP-2 are understood in detail. TIMP-1 and TIMP-2 were discovered for their erythroid potentiating activity and TIMP-2 reduces the cleavage and activation of pro-MMP-2. The TIMP's interact with MMP's by competitive inhibition of the substrate and can also form complexes with MMP's themselves leading to proteolytic destruction (Moore, 2011). Functional data on TIMP's is minimal and so this study will attempt to understand the influence of TIMP-1 in afferent activation and nociception.

Interestingly, there is evidence to suggest that inflammation can induce the endogenous opioidergic system to compensate towards an anti-nociceptive environment. Endogenous opioids are one of the most well-studied areas of pain relief in medicine. Opioids are small peptides well known for their analgesic properties. They exists endogenously playing an important role in controlling pain signals throughout the body in order to prevent excessive and chronic stimuli. They exert their inhibitory effects by binding to the three major receptors; Mu (μ), Kappa (κ), and Delta (δ). All three are G-protein coupled receptors, specifically $G_{i/o}$ where binding inhibits adenylyl cyclase thereby inactivating potassium and calcium channels. Endogenous opioids such as beta-endorphin, enkephalin, and dynorphin and their respective opioid receptors are expressed throughout the body in the central and peripheral nervous system, pancreas, immune cells, and intestinal cells (Mansour, 1994; Hughes, 2004; Zagon, 1997)

There has been much interest in the opioidergic system in recent years particularly regarding their expression and involvement during inflammatory conditions such as IBD. Studies have shown that, at least in rodent

models of colitis there is a switch in the pathology from acute to chronic colitis, with the latter producing an increase in beta-endorphin (Valdez-Morales, 2013; Verma-Gandhu, 2007). Beta-endorphin can limit the response to colorectal distension, largely due to the released beta-endorphin acting through the mu opioid receptor (MOR) on visceral afferents. This observation has also been supported at the cellular level in studies utilising patch-clamp recordings whereby acute DSS mouse colonic DRG's exhibit hyperexcitability whereby DRG's from chronic DSS mice show an increased rheobase and decrease in action potential firing due to the release of beta-endorphin (Valdez-Morales, 2013). Similar effects caused by raised beta-endorphin levels have been observed in IBS patients (Hughes, 2014). Hughes and colleagues (2014) were able to show how PBMC's isolated from IBS-C and IBS-D patients were able to attenuate *ex-vivo* nerve firing from mouse colonic afferents due to opioid release from nearby macrophages. Multiple sources for peripheral beta-endorphin release have been documented and is mostly shown to be released from circulating immune cells such as CD_4^+ T-cells, TLR_4^+ monocytes/macrophages, and neutrophils (Valdez-Morales, 2013; Sauer, 2014; Verma-Gandhu, 2007; Hughes, 2014). The mechanisms by which endogenous release of opioids may reduce the afferent activation have been suggested by various studies to include ion channels such as the transient receptor potential (TRP) channels. Opioid-based changes in the rheobase and action potential discharge in patch clamp recordings would suggest ion channels, and blocking of TLR_4-induced opioid release led to a lowering of mechanical and thermal thresholds in wistar rats, which would suggest, at least in part, ion channels such as TRP_{A1} and TRP_{V1} (Valdez-Morales, 2013; Sauer, 2014).

This chapter will therefore aim to build on the MMP data from the previous chapter on CD and examine whether the same stimulatory effect of MMP's can be observed in UC. In addition to this, functional data supports the hypothesis that the inflammatory response and by extension the nociceptive response from IBD may be influenced by a balance of pro- and anti-nociceptive mediators and so this study will investigate any potential inhibitory mechanisms that may be exploited for future therapeutic approaches.

4.1.1. The aims of this chapter are as follows:

- To understand if mechanical responses and afferent activation can be influenced by inflammatory mediators in peadiatric UC patients
- To understand if the MMP-9 and MMP-12 afferent stimulation observed in CD patients is true for UC patients
- To investigate potential inhibitory mechanisms that represent a dysregulation of pro- and anti-nociceptive changes within UC

4.1.2. TNFα mechanical responses

The expression of TNFα significantly correlated with VFh probing responses regardless of whether an overall trend for mechanical sensitivity was observed (r^2=0.78, p<0.01).

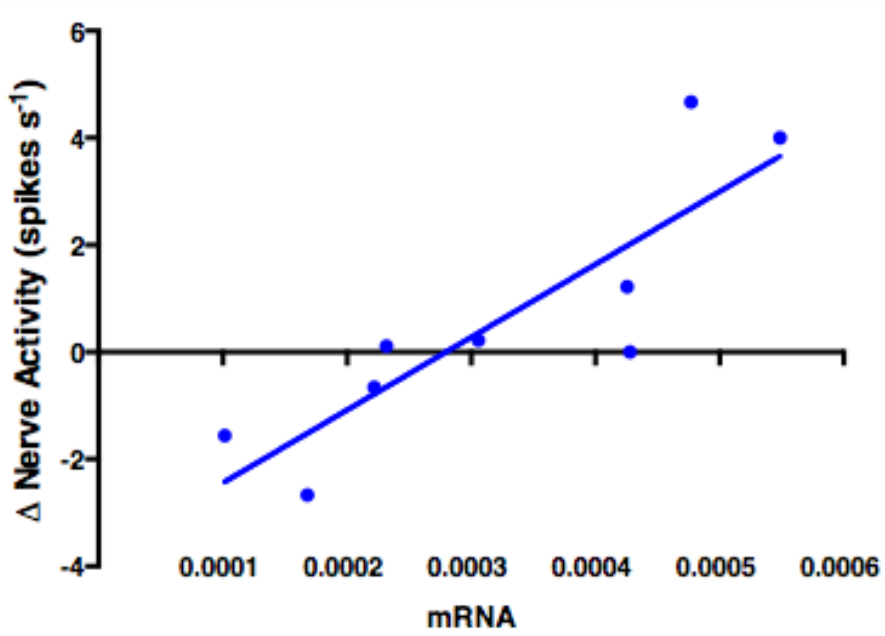


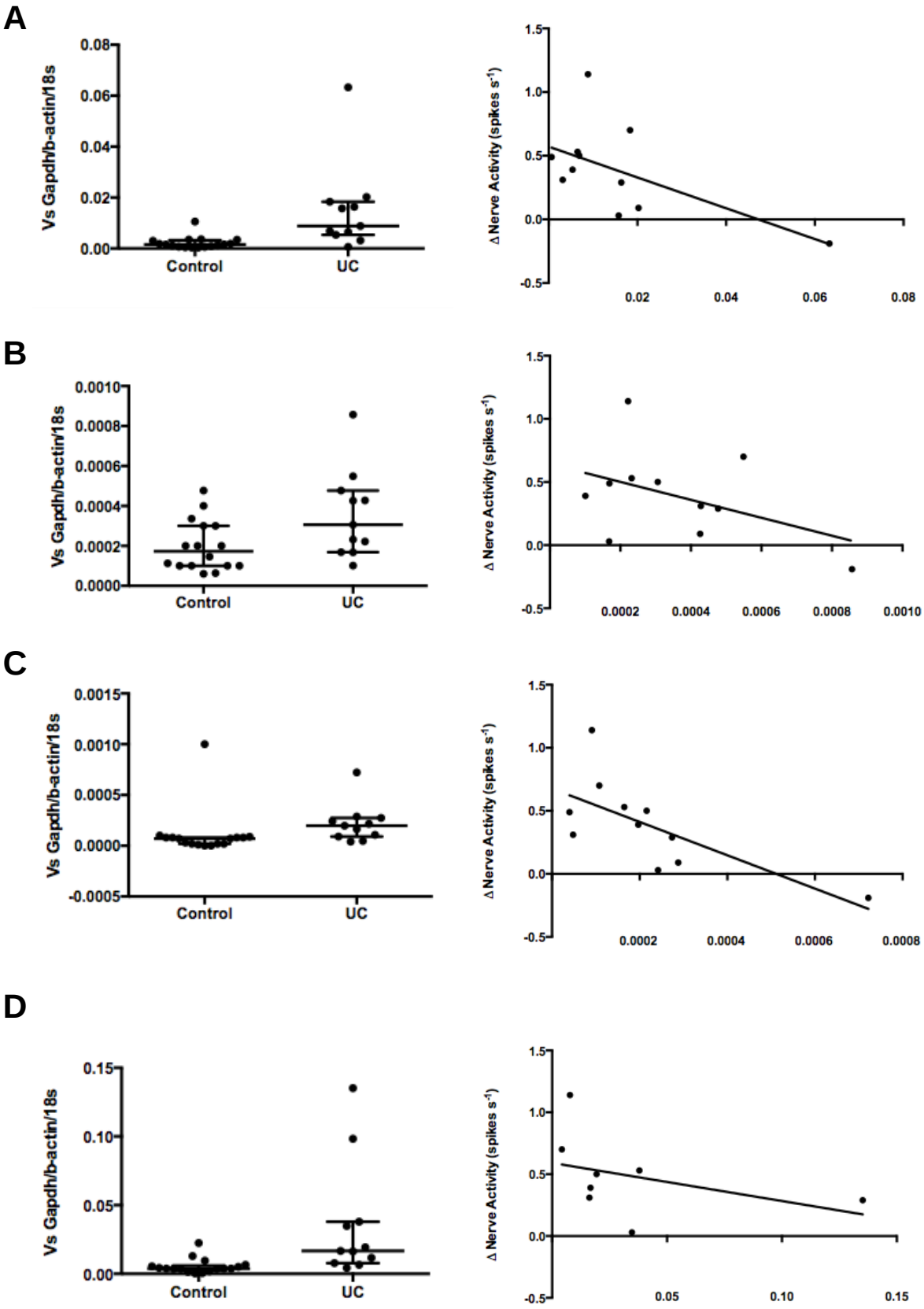
Figure 36. TNFα expression increases as VFh probed responses increase.

Biopsies from UC patients were examined for TNFα transcript levels and found to correlate significantly with afferent firing from VFh probing (p<0.01). (N= 9 , *n*=9 ; linear regression, ANOVA).

4.1.3. Cytokine expression

As UC is an inflammatory condition we assessed the expression of pro-inflammatory cytokines within the biopsy tissue. The cytokines showed median increases in expression in the UC biopsies compared with controls (IL-1β; $8.8x10^{-3}$ ($6.0x10^{-3}$ – $1.7x10^{-2}$) vs 0.038 ($3.5x10^{-3}$ – $5.4x10^{-3}$), p<0.001; TNFα; $3.1x10^{-4}$ ($2.0x10^{-4}$ – $4.5x10^{-4}$) vs $1.7x10^{-4}$ ($1.2x10^{-4}$ – $3.0x10^{-4}$), p< 0.05; IL-6; $2.0x10^{-4}$ ($1.0x10^{-4}$ – $2.6x10^{-4}$) vs $7.0x10^{-5}$ ($2.3x10^{-5}$ –

$8.0x10^{-5}$), $p<0.01$; IL-8; 0.017 ($9.7x10^{-3} – 3.6x10^{-2}$) vs 0.038 ($3.5x10^{-3} – 5.4x10^{-3}$), $p<0.001$) and also presented with a negative correlation when applied to afferent firing (IL-1β; $p=0.25$, $r^2=0.34$; TNFα; $p=0.60$, $r^2=0.19$; IL-6; $p<0.05$, $r^2=0.49$; IL-8: $p=0.13$, $r^2=0.16$).


A
Vs Gapdh/b-actin/18s
0.08
0.06
0.04
0.02
0.00
Control
UC
Δ Nerve Activity (spikes s-1)
1.5
1.0
0.5
0.0
-0.5
0.02
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Vs Gapdh/b-actin/18s
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0.0004
0.0002
0.0000
Control
UC
Δ Nerve Activity (spikes s-1)
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1.0
0.5
0.0
-0.5
0.0002
0.0004
0.0006
0.0008
0.0010
C
Vs Gapdh/b-actin/18s
0.0015
0.0010
0.0005
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-0.0005
Control
UC
Δ Nerve Activity (spikes s-1)
1.5
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0.5
0.0
-0.5
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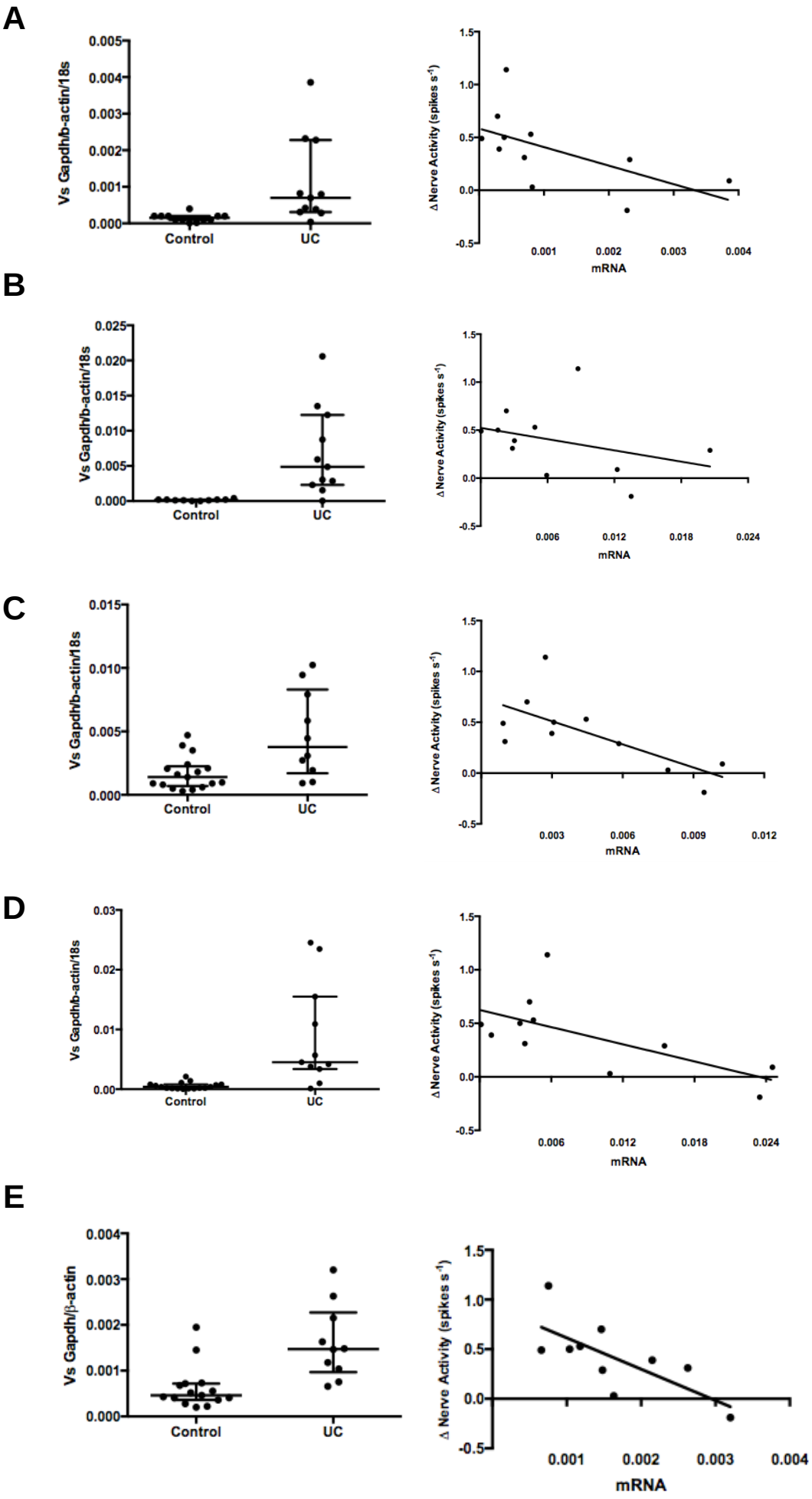
0.0008
D
Vs Gapdh/b-actin/18s
0.15
0.10
0.05
0.00
Control
UC
Δ Nerve Activity (spikes s-1)
1.5
1.0
0.5
0.0
-0.5
0.05
0.10
0.15

Figure 37. Expression of inflammatory cytokines

A significant increase in expression of **(A)** IL-1β, **(B)** TNFα, **(C)** IL-6, **(D)** IL-8, compared with controls, but a negative linear correlation occurs when coupled with afferent firing. (Control; N= 16, *n*= 16; UC; N= 9, *n*=11; Mann-Whitney, linear regression, ANOVA).

4.1.4. MMP expression

As CD showed elevated MMP expression which resulted in afferent activation, MMP's were also assessed in UC. Figure 37 shows the expression of various MMP's and inflammatory cytokines increased in UC compared with control samples (MMP-1; $7.0x10^{-4}$ ($3.4x10^{-4}$ – $1.5x10^{-3}$) vs $1.5x10^{-4}$ ($1.1x10^{-4}$ – $2.0x10^{-4}$), $p<0.001$; MMP-3; $4.8x10^{-3}$ ($2.6x10^{-3}$ – $1.1x10^{-2}$) vs $1.5x10^{-4}$ ($1.2x10^{-4}$ – $2.1x10^{-4}$), $p<0.0001$; MMP-9; $4.5x10^{-3}$ ($2.3x10^{-3}$ – $8.7x10^{-3}$) vs $1.4x10^{-4}$ ($8.1x10^{-5}$ – $2.1x10^{-3}$), $p< 0.01$; MMP-12; $4.5x10^{-3}$ ($3.6x10^{-3}$ – $1.3x10^{-2}$) ± $4.0x10^{-4}$ ($1.4x10^{-4}$ – $8.0x10^{-4}$), $p<0.0001$; MMP-19; $1.5x10^{-3}$ ($1.1x10^{-3}$ – $2.0x10^{-3}$) vs $4.6x10^{-4}$ ($3.8x10^{-4}$ – $7.0x10^{-4}$)). However, the transcripts showed a negative correlation when applied to afferent firing (MMP-1; $p<0.05$, $r^2=0.35$; MMP-3; $p=0.11$, $r^2=0.12$; MMP-9; $p<0.05$, $r^2=0.50$; MMP-12: $p=0.11$, $r^2=0.42$; MMP-19: $p=0.02$, $r^2=0.52$).


A
Vs Gapdh/b-actin/18s
0.005
0.004
0.003
0.002
0.001
0.000
Control
UC
Δ Nerve Activity (spikes s-1)
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1.0
0.5
0.0
-0.5
0.001
0.002
0.003
0.004
mRNA
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Vs Gapdh/b-actin/18s
0.025
0.020
0.015
0.010
0.005
0.000
Control
UC
Δ Nerve Activity (spikes s-1)
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1.0
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0.0
-0.5
0.006
0.012
0.018
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mRNA
C
Vs Gapdh/b-actin/18s
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0.010
0.005
0.000
Control
UC
Δ Nerve Activity (spikes s-1)
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1.0
0.5
0.0
-0.5
0.003
0.006
0.009
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mRNA
D
Vs Gapdh/b-actin/18s
0.03
0.02
0.01
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Control
UC
Δ Nerve Activity (spikes s-1)
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-0.5
0.006
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Vs Gapdh/β-actin
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0.001
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Control
UC
Δ Nerve Activity (spikes s-1)
1.5
1.0
0.5
0.0
-0.5
0.001
0.002
0.003
0.004
mRNA

Figure 38. High expression of transcripts with afferent firing.

The transcripts for MMP's in UC compared with control patients. **(A)** MMP-1, **(B)** MMP-3, **(C)** MMP-9, **(D)** MMP-12, **(E)** MMP-19 are all significantly elevated. The transcripts were plotted against the afferent firing from the supernatants and found to negatively correlate with each other. (Control; N= 16 , *n*= 16; UC; N= 9, *n*= 11; Mann-Whitney, linear regression, ANOVA).

4.1.5. Inhibitory subgroup

When it became clear that there was a negative linear relationship between inflammation and afferent firing a small subgroup of UC patients was identified as statistically different than the general UC population (figure 54). One marker of this difference was the level of active inflammation which can be categorised by levels of IL-8, one of the key initial cytokines in the inflammatory process. This subgroup (*n*=4, N=4) within the UC population in our study demonstrated significantly more IL-8 protein (UC subgroup; 141.0 ± 14.50 pg/ml; UC; 68.40 ± 14.67 pg/ml; $p<0.01$) and when the afferent firing rates were compared a significant difference was also observed (UC subgroup; 0.06 ± 0.10; spikes/s^{-1} UC; 0.53 ± 0.09 spikes/s^{-1}; $p<0.01$). The general UC group, without this subgroup potentially including inhibitory mediators, demonstrated a significantly greater level of afferent activation (0.53 ± 0.09 spikes/s^{-1}) when compared with control supernatants (control: 0.20 ± 0.03 spikes/s^{-1}; $p<0.001$). The levels of IL-8 in the subgroup were not the only mediators to be elevated when compared with the remaining UC population in our study as shown in figure 54. The UC subgroup also demonstrated increased transcriptional expression of a number of mediators including the MMP's, prostaglandin-producing enzymes (such as COX-2), and other pro-inflammatory cytokines such as IL-6 (MMP-12; 0.019 ± 0.003 vs 0.010 ± 0.003; COX-2; 0.0016 ± 5.4 x 10^{-5} vs 0.0004 ± 4.8 x 10^{-5}; IL-6; 0.00038 ± 0.00011 vs 0.00024 ± 5.9 x 10^{-5}, respectively).

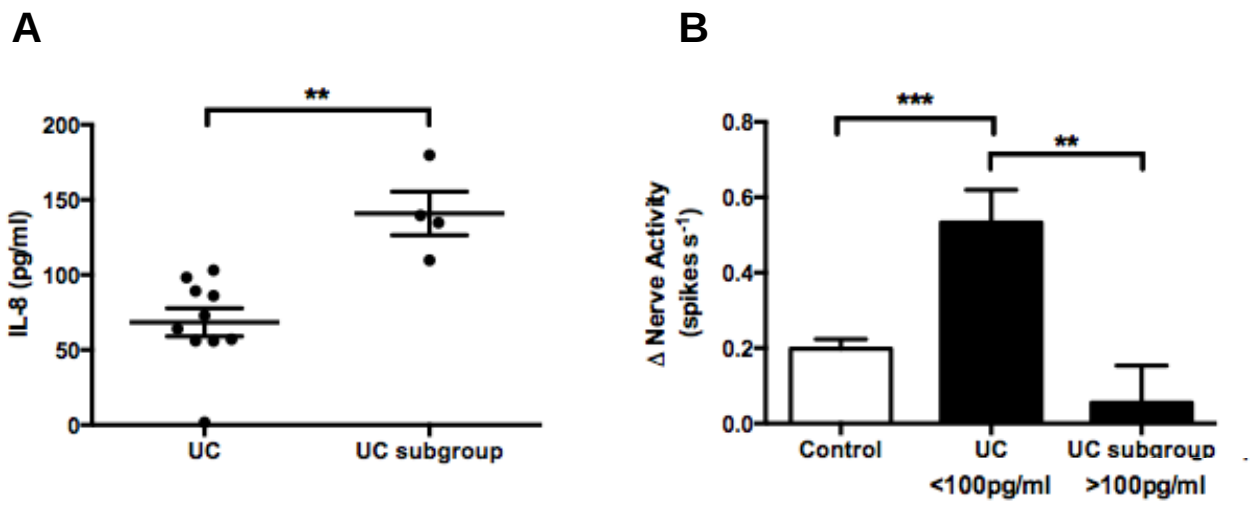


Figure 39. A sub-population of biopsies inhibit afferent activity.

(A). The protein levels of IL-8 in all the UC supernatant samples with the UC inhibitory subgroup showing constantly higher IL-8 levels. **(B).** The subgroup of UC samples show a significantly reduced level of afferent firing when the supernatants were tested on serosal afferents with IL-8 levels (pg/ml) used for differentiation. Bars represent mean ± S.E.M. IL; interleukin. Value of ** $p<0.01$, *** $p<0.001$. (Control; N= 6, *n*= 6; UC; N= 6 , *n*= 10; subgroup; N= 4 , *n*= 4; t-test).

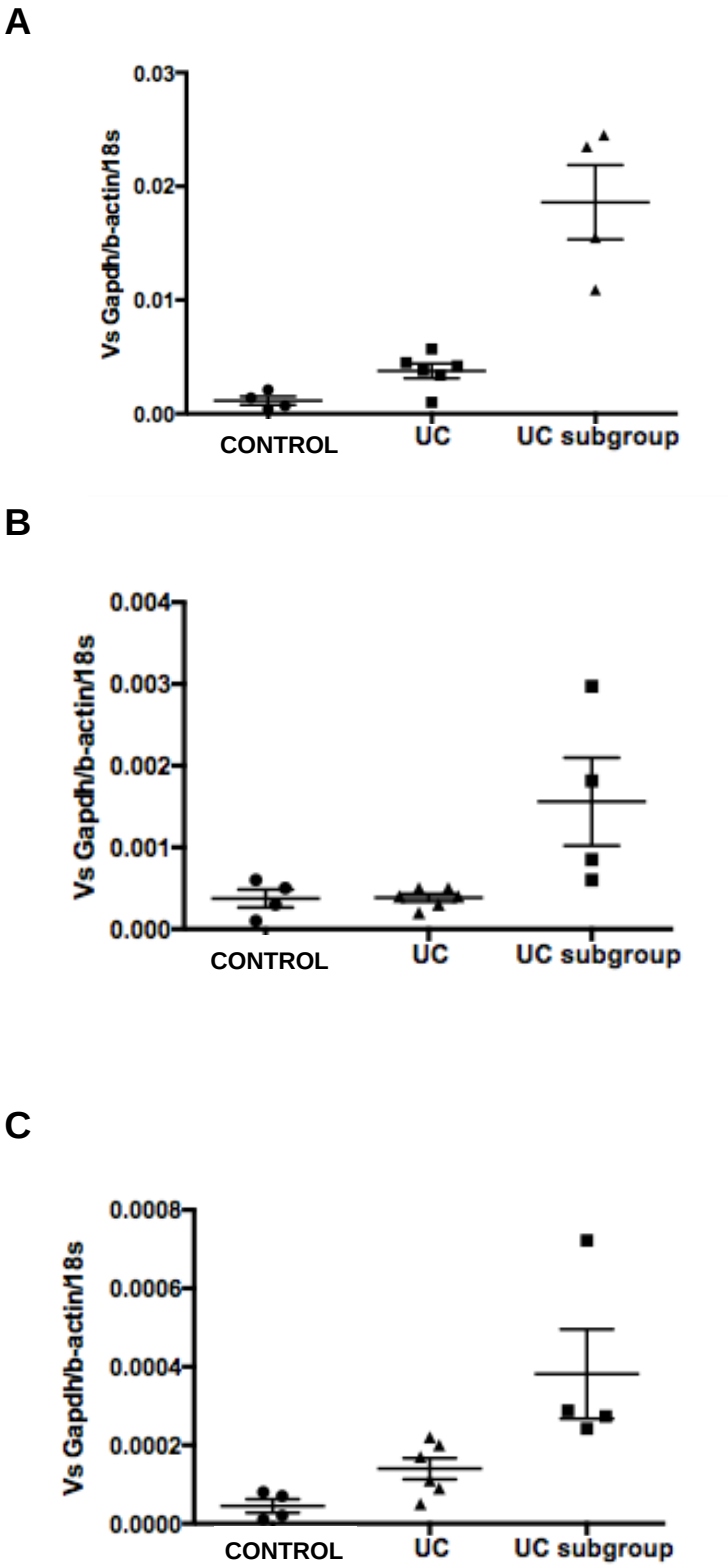


Figure 40. A sub-population of biopsies that have raised markers of inflammation.

The transcript levels for **(A)** MMP-12, **(B)** COX-2, and **(C)** IL-6 are all increased in the subgroup within the UC population compared with the control biopsies and the general UC population. (Control; N= 4 , *n*= 4; UC; N= 6 , *n*= 6; subgroup; N= 4 , *n*= 4; Mann-Whitney).

When assessing any correlations between transcript levels and afferent firing rates with the subgroup removed the negative linear correlations were abolished; MMP-1 showed p=0.15, r^2=0.96, MMP-3 showed p=0.56, r^2=044, MMP-9 showed p=0.03, r^2=0.56, MMP-12 showed p=0.36, r^2=0.08, TNFα showed p=0.99, r^2=0.71, IL-6 showed p=0.02, r^2=0.84, IL-8 showed p=0.10, r^2=0.33, with IL-1β p=0.03, r^2=0.21.

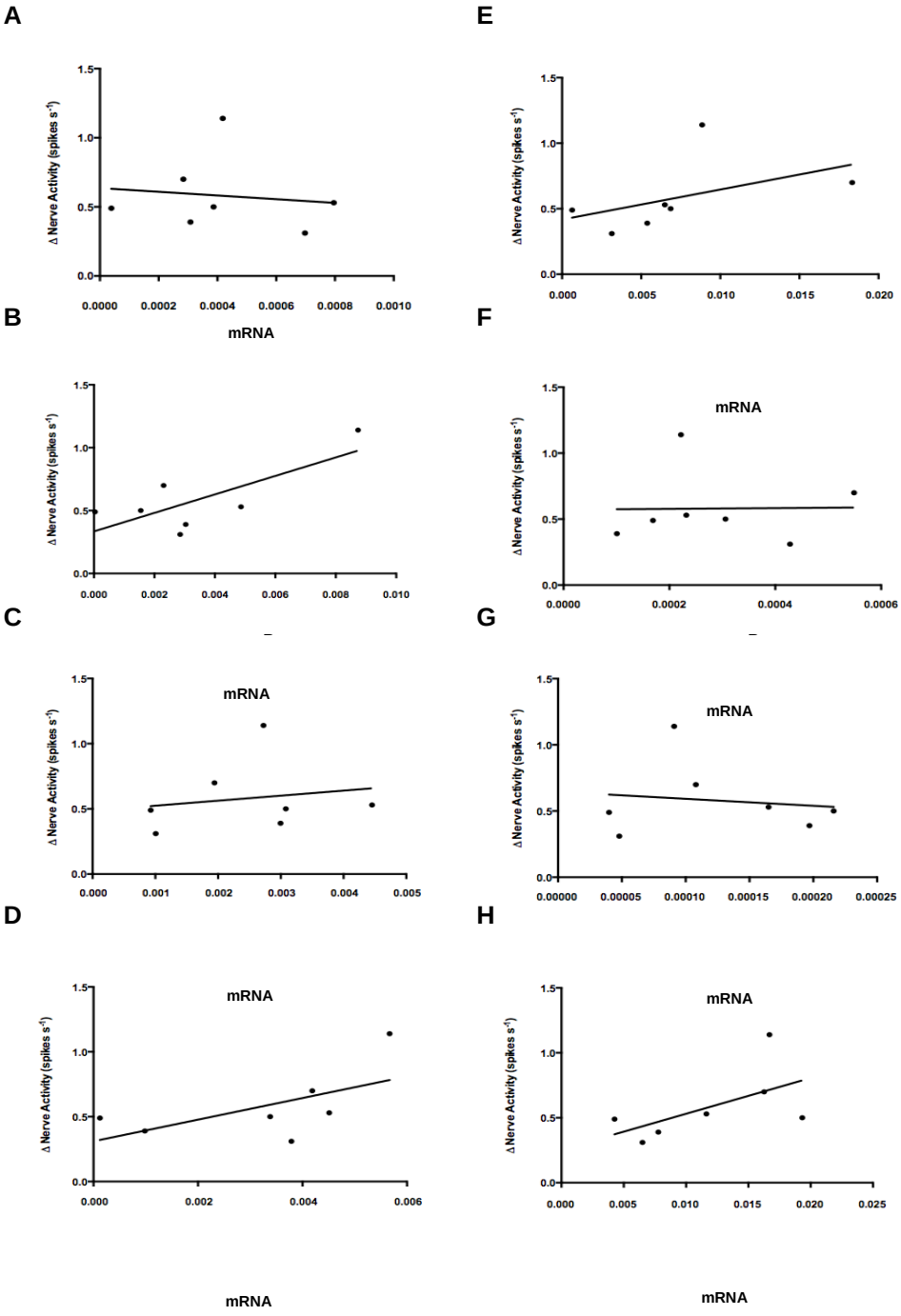


Figure 41. Transcript levels and afferent firing with subgroup removed.

With the UC subgroup removed from the data set the correlations between the transcript levels of (**A)** MMP-1, **(B)** MMP-3, **(C)** MMP-9, **(D)** MMP-12, and pro-inflammatory mediators **(E)** IL-1β, **(F)** TNFα, **(G)** IL-6, **(H)** IL-8, and afferent firing shifts to a more positive effect. The correlation between the IL-1β transcript levels and afferent firing is shown to be significant, and between MMP-12 transcript levels and afferent firing is near-significance. (N= 6 , *n*= 7 ; linear regression, ANOVA).

4.1.6. Possible inhibitory mediators

As the subgroup seemed to show an apparent inhibition of afferent firing, potential inhibitory mediator transcript levels were assessed in UC biopsies. Significant increases were found in serpin A_3, elafin, TIMP-1 and TIMP-2, when compared with control biopsies (Serpin A_3; $5.8x10^{-3}$ ($3.5x10^{-3}$ – $9.1x10^{-3}$) vs $2.7x10^{-4}$ ($2.2x10^{-4}$ – $4.6x10^{-4}$), $p<0.001$; elafin; 0.21 (0.075-0.40) vs $2.1x10^{-3}$ ($8.5x10^{-4}$ – $4.0x10^{-3}$), $p<0.001$; TIMP-1; $5.8x10^{-3}$ ($3.5x10^{-3}$ – $9.1x10^{-3}$) vs $2.7x10^{-4}$ ($2.2x10^{-4}$ – $4.6x10^{-4}$), $p<0.001$, TIMP-2; $5.8x10^{-3}$ ($3.5x10^{-3}$ – $9.1x10^{-3}$) vs $2.7x10^{-4}$ ($2.2x10^{-4}$ – $4.6x10^{-4}$), $p<0.05$, respectively). Other inhibitory mediators did not show significance (Serpin B_1; 0.13 (0.08-0.17) vs 0.11 (0.09-0.16); somatostatin; $4.4x10^{-3}$ ($3.5x10^{-3}$ – $6.6x10^{-3}$) vs $4.4x10^{-3}$ ($3.5x10^{-3}$ – $6.6x10^{-3}$); cathepsin G; $1.4x10^{-3}$ ($9.7x10^{-4}$ – $2.1x10^{-3}$) vs $1.9x10^{-3}$ ($1.2x10^{-3}$ – $2.6x10^{-3}$), respectively). Transcript levels for opioids were also assessed however many were below the lower limit of detection and could not be statistically analysed (POMC; *n*=1; pENK; *n*=1; pDYN *n*=0). When serpin A_3 was correlated with afferent firing a non-significant trend was observed ($p=0.15$, $r^2=0.16$) and only a small trend for elafin ($p=0.80$, $r^2=0.09$). When TIMP-2 was correlated with afferent firing a near-significant trend was observed ($p=0.08$, $r^2=0.44$). Interestingly, when TIMP-1 was correlated with afferent firing it was the only inhibitory mediator to show a significant correlation ($p<0.05$, $r^2=0.26$).

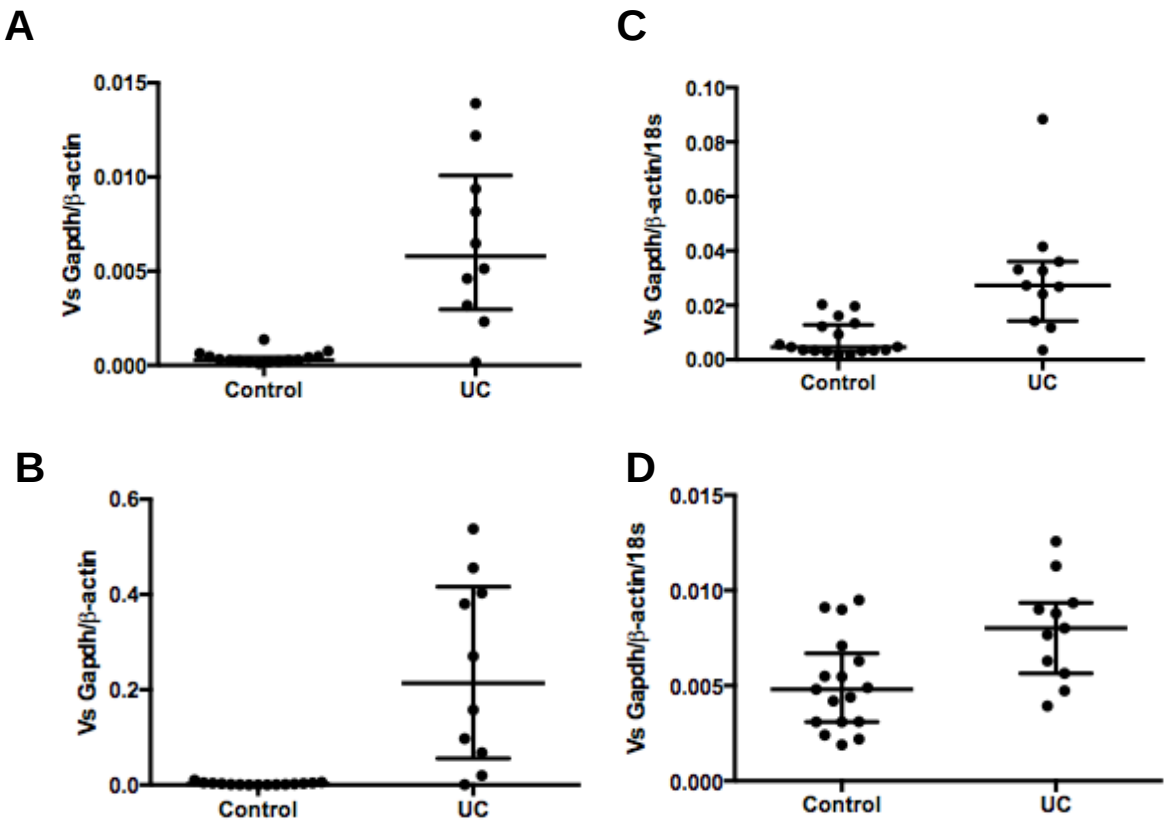


Figure 42. UC transcript levels of inhibitory mediators.

(A) The UC patients show a significantly greater level of Serpin A_3, **(B)** elafin, **(C)** TIMP-1, and **(D)** TIMP-2, compared with control biopsies. (Control; N= 15, *n*= 17; UC; N= 10, *n*= 10; Mann-Whitney).

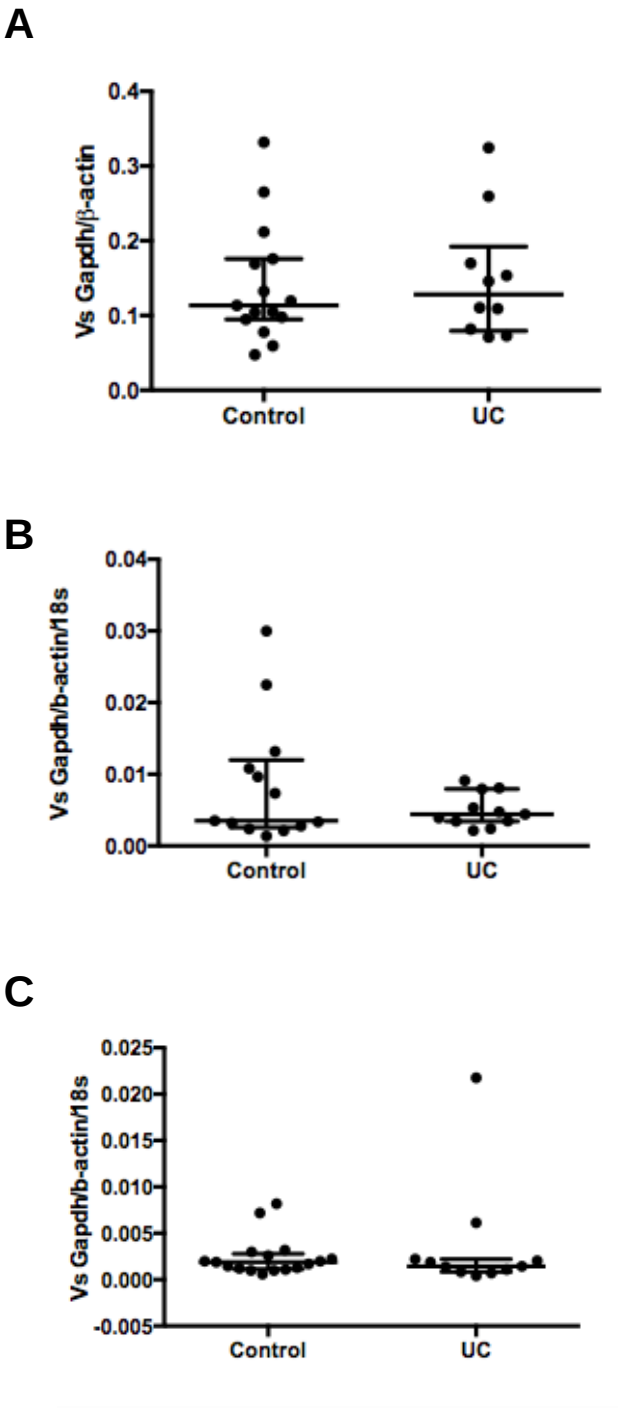
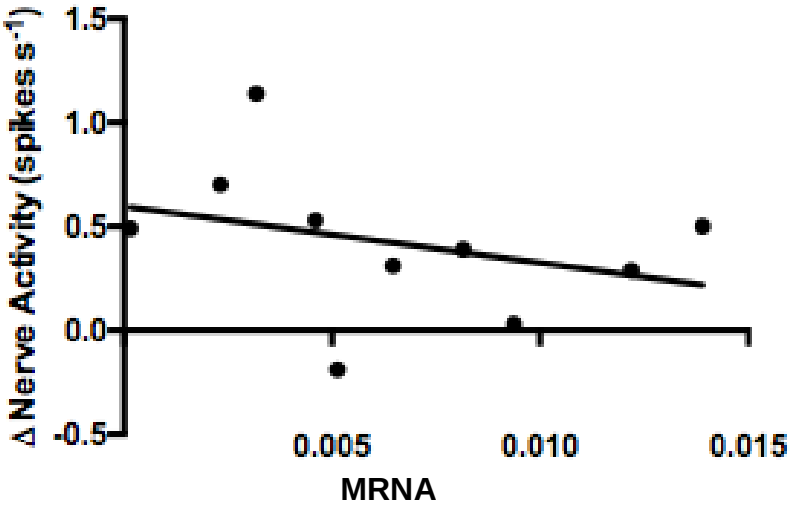


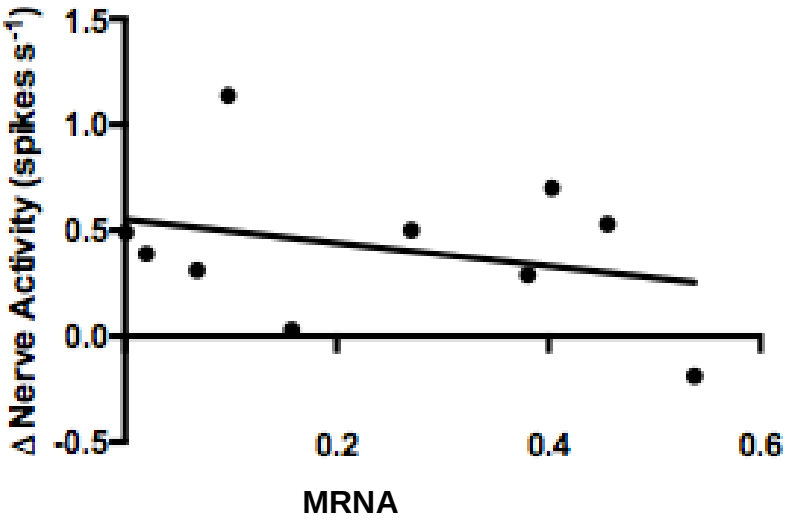
Figure 43. UC transcript levels of potential inhibitory mediators.

(A) The UC patients show a similar level of Serpin B_1, **(B)** a reduced expression of somatostatin, **(C)** and increased level cathepsin G compared to the control biopsies. (Control; N= 15, *n*= 17; UC; N= 10, *n*= 10; Mann-Whitney).

A


Δ Nerve Activity (spikes s-1)
1.5
1.0
0.5
0.0
-0.5
0.005
0.010
0.015
MRNA

B


Δ Nerve Activity (spikes s-1)
1.5
1.0
0.5
0.0
-0.5
0.2
0.4
0.6
MRNA

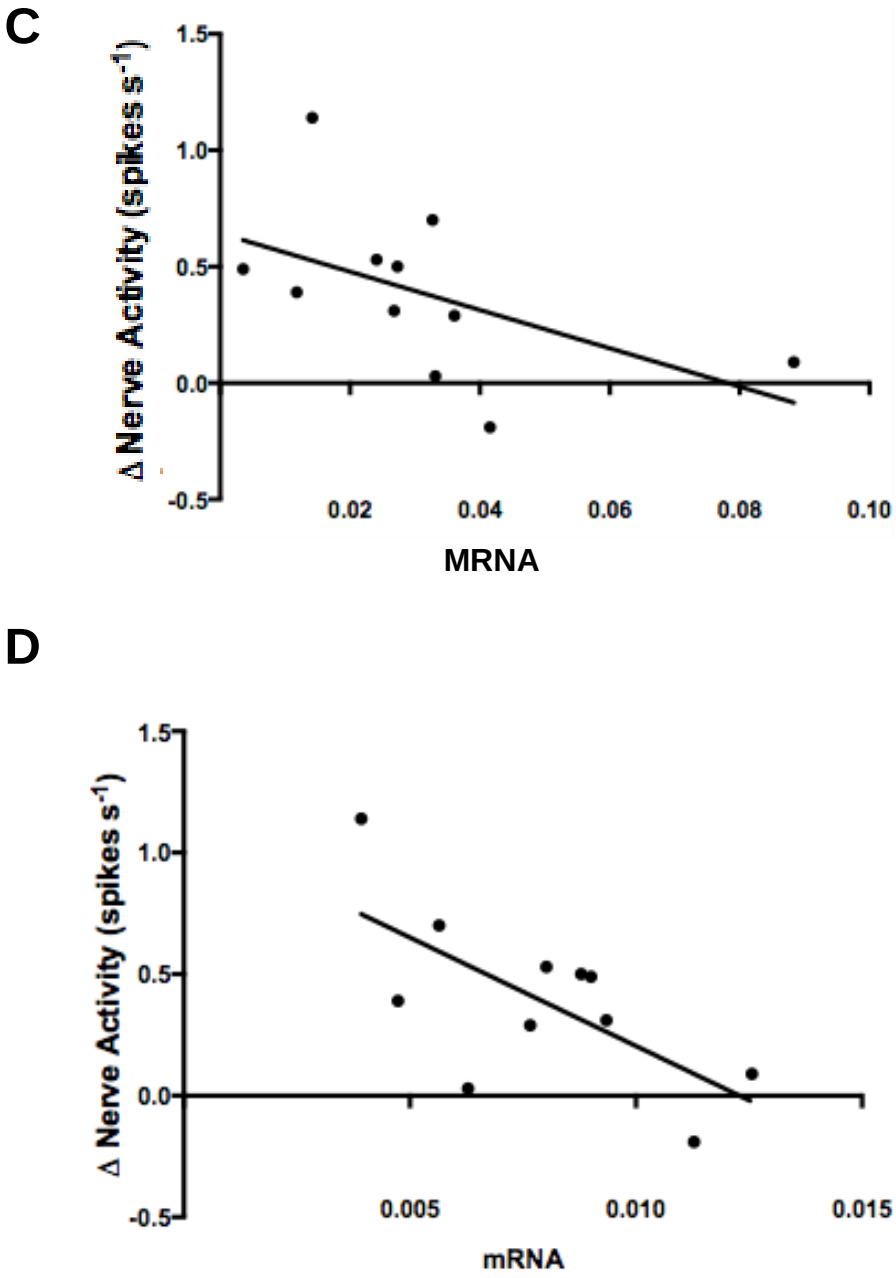


Figure 44. Inhibitory transcript levels negatively correlate with afferent firing.

(A). The transcript expression of serpin A3 shows a small but non-significant trend with afferent firing. **(B).** The transcript expression of elafin shows a very small trend with afferent firing. **(C).** The TIMP-1 levels significantly correlated with decreased afferent firing on colonic nerves ($p<0.05$). **(D).** The TIMP-2 levels correlated with decreased afferent firing on colonic nerves. (N= 10 , *n*= 11 ; linear regression, ANOVA).

4.1.7. Immune cell markers

To understand the potential source of the inhibitory mediators expression of immune cell markers were examined. As MMP-9, MMP-12, and TIMP's, are largely released from macrophages, and the influence of Treg cells on inflammatory control, markers for these cells were chosen. The transcript level for the T-regulatory (Treg) cell marker CD25 was significantly increased ($p<0.001$) in UC biopsies compared with controls ($3.3X10^{-3}$ ($3.0X10^{-3}$ – $4.9X10^{-3}$) vs $9.1X10^{-4}$ ($5.6X10^{-4}$ – $1.3X10^{-3}$), respectively). $FOXP_3$, another marker for Treg cells was similarly increased in UC biopsies compared with controls ($9.9X10^{-4}$ ($7.2X10^{-4}$ – $1.6X10^{-3}$) vs 1.8X10-4 ($1.1X10^{-4}$ – $2.6X10^{-4}$), respectively, $p<0.0001$). Two markers of macrophage presence were used to assess the UC biopsies which showed elevated levels in both CD14 and TLR_4 compared with controls (CD14; $1.2X10^{-2}$ ($8.7X10^{-3}$ – $1.3X10^{-2}$) vs $5.7X10^{-3}$ ($4.0X10^{-3}$ – $7.9X10^{-3}$), $p< 0.01$; TLR_4; $2.4X10^{-3}$ ($1.7X10^{-3}$ – $3.1X10^{-3}$) vs $1.2X10^{-3}$ ($8.9X10^{-4}$ – $1.4X10^{-3}$), $p<0.001$, respectively). IL-8, a cytokine heavily associated with neutrophil recruitment showed a strong statistically significant correlation with MMP-12, the protease largely released from infiltrating macrophages ($p<0.001$, $r^2=0.87$).

Treg release of IL-10 aids in the suppression of effector T-cells and there was a significant correlation between IL-10 and the Treg cell marker $FOXP_3$ ($p<0.05$, $r^2=0.56$).

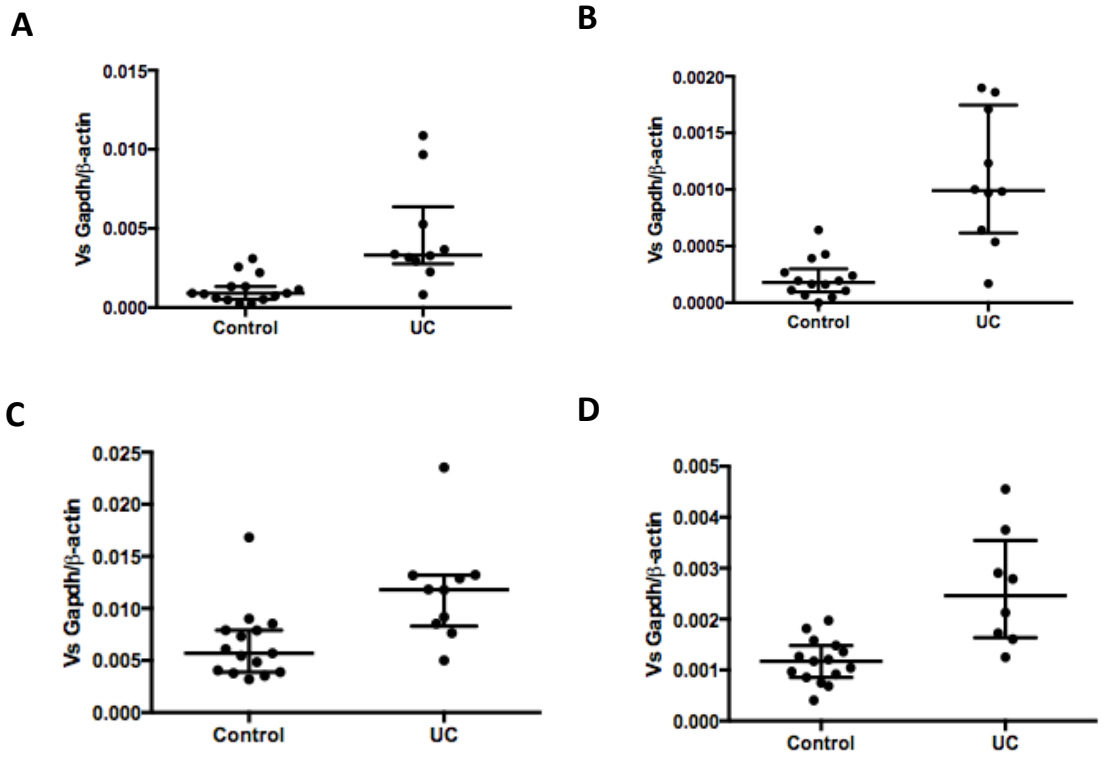


Figure 45. Transcript levels of immune cell markers in UC and control biopsies.

(A). The transcript levels for CD25, **(B)** $FOXP_3$, **(C)** CD14, and **(D)** TLR_4, all show a significant increase in UC biopsies when compared with control biopsies. (Control; N= 12, *n*= 15; UC; N=9, *n*=9; Mann-Whitney).

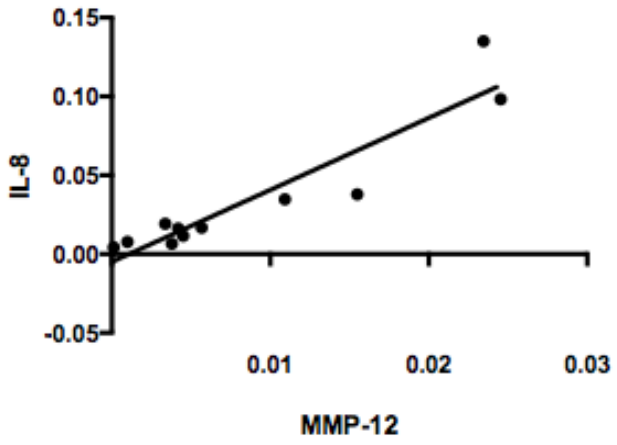


Figure 46. Neutrophil and macrophage recruitment

The expression of IL-8 and MMP-12 are strongly correlated with eachother suggesting a relationship between the recruitment of neutrophils and macrophages. (N= 9 , *n*= 11 ; linear regression, ANOVA).

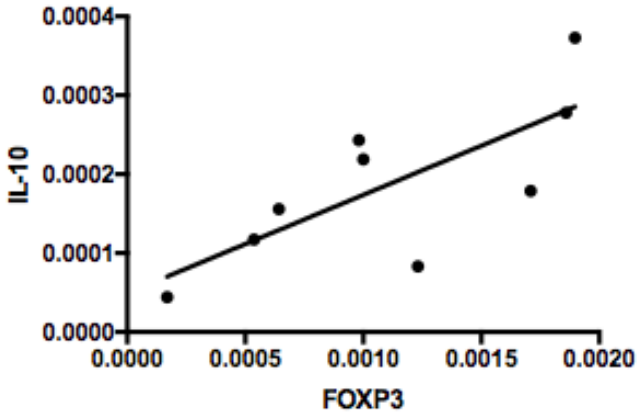


Figure 47. IL-10 expression correlates with Tregs

IL-10 expression correlates with $FOXP_3$ indicating a likely source of IL-10 comes from Treg cells helping to suppress T-effector cells. (N= 9 , *n*= 9 ; linear regression, ANOVA).

4.1.8. UC-specific IL-17 involvement to up-regulate MMP-9

As seen from our data, MMP's released from macrophages at sites of mucosal inflammation within the gut may play a strong role in abdominal pain and this may potentially be reduced through TIMP-1 inhibition. Although macrophage recruitment during inflammation may result in elevated MMP expression, this study has observed that IL-17 may play some role in increasing MMP-9 via IL-1β pathway. This was not observed in our CD data and is specific to this UC data. There was a significant correlation between IL-17 and MMP-9 ($p<0.001$, $r^2=0.88$) and between IL-17 and IL-1β ($p<0.01$, $r^2=0.27$).

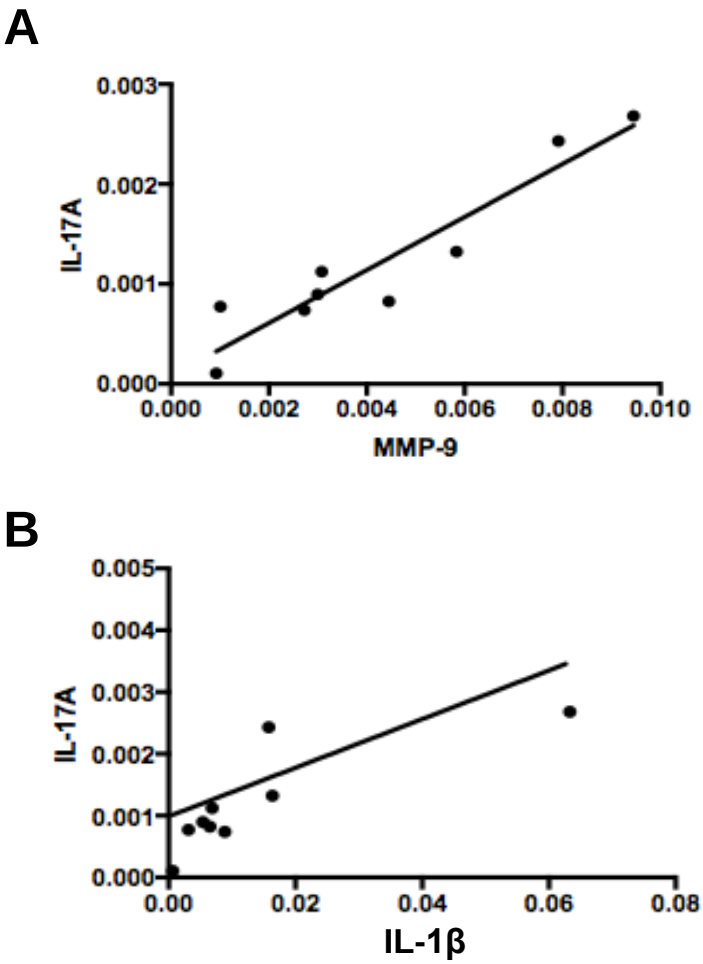


Figure 48. IL-17 correlations with MMP-9 expression

(A) A significant correlation between IL-17 expression and MMP-9 expression in UC biopsies. **(B)** A significant correlation between IL-17 expression and IL-1β expression in UC biopsies. (N= 9 , *n*= 10; linear regression, ANOVA).

4.1.9. SUMMARY OF RESULTS

- ❖ Evidence of mucosal inflammatory processes in biopsies
- ❖ Supernatants are capable of robust colonic afferent activation far greater than control supernatants suggesting a pro-nociceptive gut environment
- ❖ No changes in mechanical sensitivity were observed
- ❖ TNFα expression strongly influences VFh mechanical responses
- ❖ MMP's also highly expressed in UC
- ❖ IL-17 pathway is a potential influence on MMP-9 expression
- ❖ Greater inflammation leads to a reduction in afferent firing due to presence of an inhibitory subgroup, specific to UC
- ❖ Inhibitory subgroup strongly influenced by TIMP-1 and TIMP-2, but also serpin A_3 and elafin
- ❖ Protease inhibition likely responsible for decreases in afferent firing and could be a potential therapeutic target

4.2. DISCUSSION

4.2.1. Overview

Ulcerative colitis is a severe inflammatory condition affecting the colon and rectum (table 5). One of the most debilitating symptoms is severe abdominal pain and regardless of inflammatory resolution, patients entering clinical remission often report severe episodes of abdominal pain (Jonefjäll, 2015; Takeuchi, 2006). In this chapter, we have aimed to build on evidence from chapter 3 which demonstrated an increased expression of MMP's where MMP-9 and MMP-12 both appeared to directly and indirectly result in nociception.

4.2.2. Afferent response to supernatants

As performed in the previous chapter with CD biopsy supernatants, the potential of mediators within the supernatants to activate colonic afferents was assessed. Supernatants were added to mouse serosal layer receptive fields in the distal colon using whole-nerve electrophysiological recordings. UC supernatants produced robust responses in firing significantly greater than controls, demonstrating a colonic environment from UC patients which represents a more nociceptive and potentially painful environment than patients without the disease (figure 13, table 9).

As with the other supernatants from this study, UC supernatants produced robust responses in approximately half of the afferents tested (53%). Pro-inflammatory and pro-nociceptive mediators released from colonic mucosa in UC patients has been reported in numerous studies and several of these mediators are capable of directly directly stimulating sensory neurons. For example, IL-1β, IL-6, IL-17, ATP, and PGE_2, are elevated in UC patients and contribute to chronic inflammation by regulating further cytokine and chemokine release and *in vitro* studies demonstrate their ability to generate action potentials (Burnstock, 1996; Obreja, 2002; Rush & Waxman, 2004; Brenn, 2007; Richter, 2012; Hughes, 2013). However, this is the first study to show biopsy supernatants from UC patients stimulate colonic afferents demonstrating a pragmatic approach to studying abdominal pain in UC.

In addition to a direct ability of the supernatants to cause an increase in afferent firing, the mechanical responses were also tested to understand the capacity for changes in mechanical sensitivity due to supernatant mediators (figure 14). The variability of VFh probing could be suggested as a cause of this result and further examination of mechanical responses using phasic distensions may provide a more sensitive

measure for future studies, as the VFh probing in this study may have been below the mechanical threshold to observe changes in neuronal excitability from only a short incubation on the receptive field. Perhaps a longer exposure would have meant the development of a greater opportunity for membrane receptors and ion channel up-regulation or activation. Contrary to mechanical responses however, and consistent with current literature, when analysing the transcript levels of TNFα in UC biopsies, there was a strong positive correlation with probed responses (figure 36) (Hughes, 2013). As the TNFα transcript levels increased, the ability of the supernatants to enhance the mechanical response post-supernatant incubation was also increased. TNFα has been implicated in mechanical sensitivity of afferent endings whereby direct application can lead to an increase in VFh probed response. This has been suggested to elicit mechanical allodynia through TNF_{R1} activation and modulation of $Nav_{1.8}$ channels via p38 MAP kinase phosphorylation (Ibeakanma, 2009). Whereas Hughes *et al.* (2013) observed protein levels of TNFα to be associated with mechanical responses in colonic afferents, this study only observed a correlation with TNFα expression and VFh probing and so future studies directed at understanding the mechanism of action in UC patient samples would be of benefit. TRP_{A1} antagonists have also demonstrated an ability to attenuate inflammation-induced mechanical hypersensitivity, where TNFα plays a dominant role, and TRP_{A1} has also been associated with PAR_2 coupling in afferent activation and this receptor/ion channel coupling could potentially influence TNFα associated mechanical changes in this study (Zhao, 2015; Grant, 2007).

Surprisingly, changes in mechanosensitivity are not observed from supernatants but only TNFα transcript expression, which could be for a number of reasons including the combination of mediators within the supernatant which may limit or change the overall VFh responses thereby minimising the observable effect of TNFα, and in addition this study may also be susceptible to low numbers of replicates. Further investigation to assess TNFα alone on the receptive fields may produce a clearer understanding of the mechanical response in future studies. Undoubtedly, further studies utilising TNFα blocking compounds within supernatants would be able to investigate this mechanism further.

4.2.3. Cytokine and MMP expression produced a negative correlation

Further assessment of cytokine transcript expression in UC biopsies revealed a significantly greater level of

expression compared with control patients for the pro-inflammatory cytokines IL-1β, TNFα, IL-6, and IL-8 (figure 37, table 8). However, when this expression was coupled with afferent firing there was a consistent negative linear correlation suggesting that greater inflammatory processes within the tissue would actually result in less afferent activation. This is in direct contrast to current literature demonstrating inflammatory mediators result in nociception and data observed from CD patient biopsies from chapter 3, where greater inflammatory processes often correlated with greater levels of afferent firing (Burnstock, 1996; Obreja, 2002; Rush & Waxman, 2004; Brenn, 2007; Richter, 2012; Hughes, 2013).

To understand if this phenomenon was unique to pro-inflammatory cytokines, or represented a global effect in UC pathology, MMP's were also assessed. Our data suggests that CD supernatant afferent activation is influenced by MMP's and therefore it is expected that a similar pathology may present itself in UC. All 5 MMP's analysed (MMP-1, -3, -9, -12, -19) were significantly raised compared with controls suggesting a similar protease environment as the CD biopsies (figure 37). In accordance with this, correlations between MMP's and afferent firing was analysed to understand any relationship between the two. Surprisingly, all MMP's also showed a strong negative correlation with afferent firing suggesting that MMP's somehow reduced or inhibited the afferent from launching action potentials (figure 38, 40).

Further investigation into this observation revealed that four biopsies had a dominant effect on the analysis of the UC biopsies (figure 39, 40). The four biopsies reported the highest levels of the inflammatory marker IL-8, but when the supernatants were added to colonic afferents, they resulted in a significantly reduced level of afferent firing when compared to the remainder of the UC group, suggesting an inhibitory profile from a small number of patient biopsies and that in this subpopulation of patients, as the disease severity increased, and pro-inflammatory and pro-nociceptive mediators increased, they were likely to result in active inhibition of nociceptive responses at the peripheral ending (figure 42, 43).

Interestingly, when this subgroup was removed from the analysis, the negative correlations observed previously were abolished, where in some instances, such as MMP-1 and MMP-12, the transcript levels now positively correlated with firing rates. These correlations suggest that MMP-12, and possibly MMP-1 may also serve as potential mediators of nociception and pain in patients with UC, which is largely in agreement with our findings from CD patients.

Consistent with the hypothesis that a subgroup of biopsy supernatants may contain mediators to actively inhibit afferent firing during severe inflammation, biopsies were next assessed known inhibitory mediators. The total UC group was analysed for a variety of known inhibitory mediators (serpin A_3, serpin B_1, somatostatin, elafin, cathepsin G, TIMP-1, TIMP-2, POMC; the precursor for β-endorphin, pro-dynorphin, pro-enkephalin) and it was observed that the transcript levels for serpin A_3, elafin, TIMP-1, and TIMP-2, were significantly greater than those of the control biopsies (figure 42).

4.2.4. Inhibitory mediators

Serpin A_3 is a member of a superfamily of protease inhibitors which has a targeted role in inflammation where it helps to regulate serine protease levels. Serpin A_3 is found in neutrophils and mast cells and there is evidence that it has a protective role against neuropathic pain and mechanical allodynia due to the inhibition of neutrophil elastase and downstream production of MMP-9, also linked to neuropathic pain (Vicuña, 2015). This study demonstrates that UC patients have a significant increase in serpin A_3, although when a comparison with afferent firing was made, no significant correlation became apparent (figure 43) suggesting that it may not heavily influence afferent activation from UC supernatants, but may contribute to a reduction in overall protease involvement.

Expression of elafin was also significantly increased compared with control samples (figure 42). Elafin is a serine protease inhibitor but also shares anti-microbial properties (Schalkwijk, 1990; Simpson, 1999). Released from epithelial cells and immune cells such as macrophages and neutrophils, the increased expression could therefore result in a reduction of protease activity (including but not exclusive to MMP's) which may result in a reduced afferent response to supernatants (Mahaila & Tremblay, 2001). No statistically significant correlation with afferent firing was observed. Therefore, similar to the contribution of serpin A_3 discussed above, there may be a contribution towards a reduced protease environment and greater protease control thereby limited afferent firing and nociception. Literature documenting the release and functional response of beta-endorphin from macrophages in DSS mouse models of inflammation, or adult IBS-C and IBS-D patient populations, have previously shown the anti-nociceptive benefit of this inhibitory protein, however this is the first study to investigate inhibitory mechanisms in UC paediatric patients (Valdez-

Morales, 2013; Hughes, 2014). This current study suggests that while not strongly associated with a reduction in afferent firing in UC patients, pragmatically, there is an overall contribution which is likely to involve a combination of protease inhibition which is necessary for an attenuated afferent response.

As discussed in chapter 3, MMP's appear to play a role in afferent activation within IBD supernatants. In consideration of this, the endogenous inhibitors of MMP's, the TIMP's, were assessed in UC biopsies and found to be significantly increased compared with control samples. Evidence from literature for the expression of TIMP-1 in UC in concert with high MMP levels suggests a dysregulation between the TIMP's and MMP's may explain, at least in part, a basis for abdominal pain (Wang, 2009; Jakubowska, 2016). As functional data on the inhibitory performance from TIMP-1 in this study is unavailable, this process of dysregulation within the current supernatants is open to speculation. However, we did observe a statistically significant correlation between greater TIMP-1 and TIMP-2 expression and reduced afferent activity suggesting that the absence of TIMP-influence in biopsy supernatants may result in greater nociception and potentially abdominal pain (figure 44). Currently, there are no studies assessing TIMP expression with functional data supporting their influence in abdominal pain. This study is the first to identify a small number of paediatric UC patients that have elevated TIMP-1 and TIMP-2 expression which correlates with afferent response suggesting that TIMP-1 and TIMP-2 strongly suppress protease activity to reduce afferent firing and therefore likely abdominal pain in patients. Further functional data supporting this would be able to identify optimal levels of localised TIMP's, and therapeutically, TIMP-1 and TIMP-2 could be administered to specific regions of high protease activity, and data from this study suggests that MMP-9 and MMP-12 would be idenifty as targets of interest.

Several other potential inhibitory mediators were also measured to understand if the inhibitory effect contained multiple components. Although many mediators assessed within the biopsy samples did not show elevated expression compared with control samples, this expression is beneficial to understanding the components of the UC biopsy supernatant.

The opioidergic system was assessed and levels of pro-dynorphin, pro-enkephalin, and POMC were measured in biopsy samples (figure 42). Opioids are endogenous peptides that play an important role in in the control of pain processing and are expressed throughout the body and nervous system, with particularly

high concentrations found in pancreas, immune cells, and intestinal cells (Mansour, 1994; Hughes, 2014; Zagon, 1997). Multiple sources for peripheral β-endorphin release have been documented to demonstrate its release from circulating immune cells such as $CD4^+$ T-cells, TLR_4^+ monocytes/macrophages, and neutrophils (Valdez-Morales, 2013; Sauer, 2014; Verma-Gandhu, 2007; Hughes, 2014). Although endogenous opioids have been shown to increase during chronic inflammation, where elevated β-endorphin levels observed in rodent models of colitis have been associated with the progression of acute to chronic colitis, this study failed to detect expression and therefore were likely to have no influence on afferent activation suggesting that they may not influence peripheral mechanisms of nociception in UC, but this does not disregard potential influences within the spinal cord and CNS (Valdez-Morales, 2013; Verma-Gandhu, 2007). However, the translation between transcript expression and protein expression may be difficult to interpret and it is likely that immune cell infiltration with greater proximity to serosal layer nociceptors in patients may have been missed with mucosal biopsy samples in this study.

Serpin B_1, somatostatin, and cathepsin G, are all known for their actions in reducing neuro-immune interactions, such as limiting the release of IFNɣ and macrophage mediator release at peripheral nerves but were not statistically altered compared with control samples and no relationship with afferent firing was observed which suggests that there is a minimal influence of these inhibitory mediators in UC patients (Uchiyama, 2012; Szolcsányi, 1998).

Regardless of the patient size of the subgroup, indications that serpin A3, elafin, TIMP-1 and TIMP-2, may all contribute to reducing afferent activity is intriguing. The TIMP-1 and TIMP-2 demonstrated a statistically significant correlation between expression and afferent firing suggesting that the involvement of TIMP inhibition, and as an extension, MMP activity, is a driving mechanism behind afferent inhibition. However, it must be taken into account that a combination of protease limiting mediators such as these likely work together to limit protease dysregulation and protease-driven afferent activation. Therefore it may be beneficial to understand the protease involvement in UC with a view to exploit greater TIMP-1 and TIMP-2 activity to reduce nociception and abdominal pain.

With the identification of an inhibitory subgroup within the UC patients, it is important to understand any potential sources of these mediators (figure 46). Neuro-immune interactions in UC can provide an important

pathway into understanding peripheral afferent activation and nociception, therefore immune cell levels were assessed in biopsies. It was found that increased expression of both CD25 and $FOXP_3$, markers of T-regulatory cells (Treg), were observed in all UC samples (figure 45). Tregs are a subpopulation of T-cells that suppress the immune system and work to restore homeostasis during chronic inflammation. The Tregs suppress the expansion of effector T-cells largely by releasing IL-10 and TGF-β, and data from this study shows a positive correlation between IL-10 and $FOXP_3$ suggesting this process may be occurring in our UC biopsies (figure 47). IBD has previously been shown to reduce Tregs which is in part responsible to chronic inflammation and the transfer of Tregs in healthy mice to inflamed mice demonstrates a partial reversal in chronic inflammation (maul, 2015; Martin, 2004). Hence, it is possible that Treg dysregulation may not be entirely initiated in children which would support data in this study showing elevated Treg cells, and this suggests that early intervention in children could have potential to minimise chronic inflammatory states in the future although further studies to understand this would be necessary.

The macrophage markers were significantly increased in UC samples suggesting macrophage infiltration into mucosal tissue. The $CD14^+$/ TLR_4^+ macrophages are a key source of pro-inflammatory cytokines such as IL-1β, TNFα, IL-6, IL-8, IL-23, INF-ɣ, and also MMP's and TIMP's. The release of TIMP-1 and TIMP-2 appears to be a controlling factor in reducing the afferent activity and one potential source of this is likely to be infiltrating macrophages. Interestingly, and unique to UC samples in this study, a strong correlation between IL-8 and MMP-12 expression was observed (figure 46), suggesting that there is a linear relationship between neutrophil and macrophage recruitment in UC. The implications of this suggests a reason for the high anti-protease environment in the supernatants, as serpin A_3 and elastase are released from both neutrophils and macrophages, and may explain why this was not observed in CD samples. This study hypothesises that elevating TIMP's and other protease inhibitory will potentially be beneficial in attenuating afferent firing and nociception.

IL-17 is a classical Th_{17} cytokine released from numerous immune cells including monocytes and neutrophils and has more recently gained interest for its potential role in experimental models of pain. It has recently been reported that IL-17 may influence MMP-9 levels via an integrated pathway involving IL-6 and IL-1β cytokines (Shibata, 2014). In view of this a relationship between IL-17 expression and inflammatory

mediators was assessed and a linear relationship between IL-17 and MMP-9 was observed (figure 48). In addition, IL-17 also significantly correlated with IL-1β expression suggesting that it may be influencing MMP-9 activation via the IL-6R-IL-1β mediated pathway reported recently (Shibata, 2014). Although this is not conclusive evidence for such a pathway, it does warrant further investigation to understand the benefit of IL-17 as a target for MMP-9 related nociception.

4.2.5. Conclusion

In conclusion, an acute incubation of human paediatric UC biopsies produced supernatants with the capacity to model one aspect of UC, the nociception and potentially visceral pain in patients. The supernatants stimulate nociceptors to a much greater extent than from control patients with no inflammation or abdominal pain. For the biopsies that do cause direct activation, it seems likely that as with CD, the MMP's, potentially MMP-1 and MMP-12 play a major role. Interestingly, with UC biopsies in this study, a subgroup emerged whereby high levels of TIMP-1, TIMP-2, and elafin were coupled with low afferent firing suggesting that these protease inhibitors should be investigated further to understand their potential as therapeutic targets for visceral pain in UC.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSION

This study assessed the potential use of biopsies from patients with abdominal pain in disease (FAPS, CD, UC) in order to model nociception in mouse colonic afferent extracellular recordings. It was found that a short incubation (1 hour) of the biopsies was enough to ensure a supernatant contained pro-nociceptive mediators to a high enough concentration in order to elicit afferent firing. The supernatant and biopsy were then mined for potential mediators involved in nociception with a view to understanding identified mediators as potential sources of abdominal pain in patients. This study describes the ability of proteases, specifically MMP-9 and MMP-12, to elicit direct afferent firing in both rodent and human colonic afferents. In addition, this study found they could modulate afferent responses to mechanical or chemical stimuli. Finally, this study identified that protease inhibition is likely to be effective as a targeted pathway for minimising nociception and future studies should adopt protease inhibition for abdominal pain.

To understand the effect a biopsy supernatant would have on serosal layer colonic afferents they were applied over receptive fields for a short time. In all patient phenotypes, supernatants robustly activated colonic afferents to a greater level compared with control supernatants suggesting disease pathology results in a gut environment which is pro-nociceptive. Between 50-60% of supernatants generated responses which is similar to response rates observed in recordings of submucous and myenteric neurons when adult IBS supernatants were added (Buhner, 2009; 2012). This may be a result of the afferent subtype and expression of specific metabotropic receptors or ion channels needed to supernatant activation, or a result of supernatant composition. It is plausible that both circumstances will play a role.

Abdominal pain in patients is often associated with movement of food through the bowel thereby identifying mechanical sensitivity as key feature of pain in GI diseases. This has been replicated in various animal models of visceral hypersensitvity and so this study looked at changes to receptive field mechanical sensitivity using 1g VFh probing before and after supernatant incubation (Spiller, 2004; Matsumoto, 2012; Deiteren, 2014; Qi, 2016). However, overall there appeared to be only marginal changes in mechanical sensitivity in FAPS, CD, or UC, supernatants and did not warrant conclusive evidence of mechanical changes from biopsy supernatants. This is likely due to the variability of VFh probing which may lend itself to statistical error in relatively small patient studies such as this one. Increasing replicates or including additional VFh probe weights would be a consideration for future studies.

Evidence throughout literature and data from this laboratory suggests that various pro-inflammatory mediators contribute not only to the progression and prolonged inflammatory response, but also act as pro-nociceptive mediators to nociceptors (Corvera, 1999; Binshtok, 2008; Reed, 2003; Taylor, 2010; Hughes, 2013; Balemans, 2017). Transcript levels of various mediators including tryptase, 5-HT, histamine, IL-1β, IL-6, COX-1, and COX-2 for PG's, were assessed compared with control samples. Although transcript levels were often elevated in patient biopsies (only limited changes were observed in FAPS patient biopsies), none significantly correlated with afferent firing and can only be assumed to play a minor role in afferent activation within this study. It is important to consider that the short incubation times for the supernatant could mean that protein levels of mediators had not reached concentrations high enough to elicit strong effects on colonic afferents and so this study does not necessarily offer an alternative to current literature. In consideration of this, it is possible that other mediators play a much greater role in supernatant activation and nociception.

Mediator expression analysis in FAPS biopsies did not reveal any significant differences compared to control biopsies which may result from the pathology of the mucosa where the biopsy was taken, rather than the full thickness of the colon wall in FAPS patients. However, another approach to reducing afferent activation form FAPS was assessed by observing the influence of the $TRPV_4$ ion channel in supernatant responses. $TRPV_4$ a non-selective 6-transmembrane cation channel with a preference for calcium ions and there is evidence to suggest its involvement in somatic pain where it acts as a high-threshold mechanoreceptor in addition to mediating allodynia and hyperalgesia (Suzuki, 2003; Sipe, 2008; Cenac, 2008; Vergnolle, 2010; Alessandri-Haber, 2004; Alessandri-Haber, 2005). $TRPV_4^{-/-}$ mice were used to understand if FAPS biopsy supernatants would retain their ability to stimulate colonic afferents. This study found $TRPV_4^{-/-}$ afferent responses to be significantly reduced compared to $TRPV_4^{+/+}$ and C57BL/6 WT mice. As the supernatants were pooled together it is unlikely that variability between supernatant composition would have an effect, therefore this study suggests that the $TRPV_4$ ion channel is needed for nociceptor signalling and the suppression of this ion channel could result in a reduction in afferent signalling in FAPS patients.

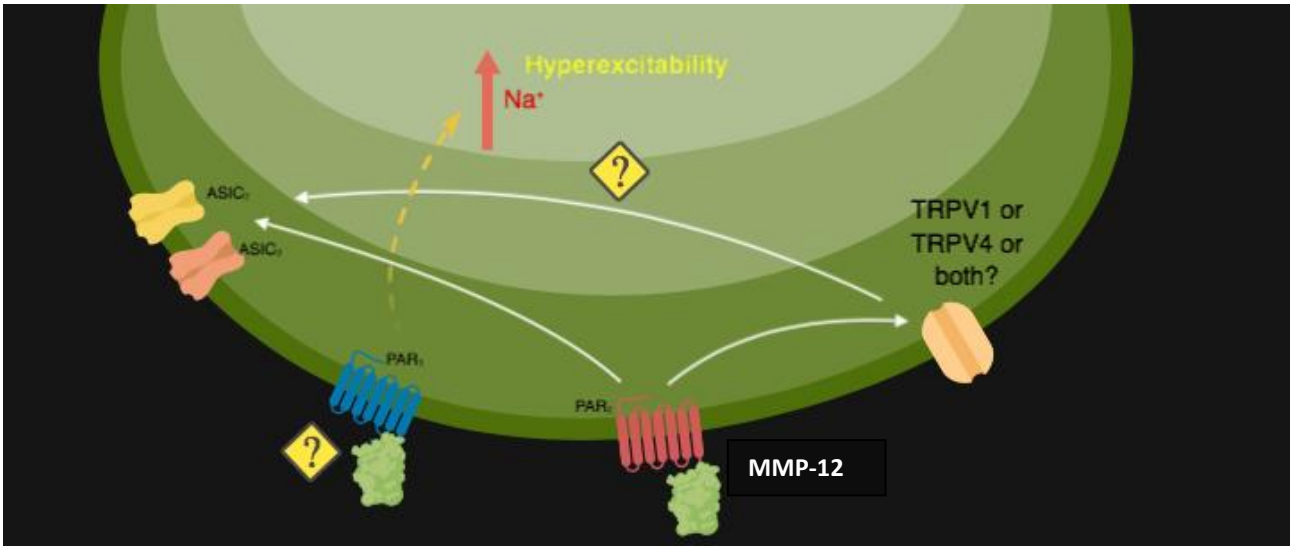


Figure 49. Potential influence of MMP-12 on mechanical sensitivity

Mechanisms are currently unknown and have yet to be investigated, however, possible interaction with PAR_2 or PAR_3 may encourage signaling pathways which phosphorylate ASIC channels and TRP channels influencing the membrane threshold for further stimulation. The different distribution of ASIC channels on serosal and mesenteric afferents may explain the contrasting effects observed in this study

Although pro-nociceptive mediator expression was not elevated in FAPS biopsies, the inflammatory environment within IBD did present with significant changes compared with control biopsies. One of the most striking and important discoveries from this study was the enhanced expression of MMP's in both UC and CD, and the correlation of MMP-12 with afferent firing in CD. When MMP-12 was applied exogenously to both mouse and human colonic afferents, it resulted in a robust increase in firing. This endorses the results from the comparison between expression levels and afferent response and we also demonstrated that MMP-12 can directly stimulate native channels in human nociceptors in much the same manner as mouse afferents. It also suggests that mouse colonic afferents represent a suitable surrogate for human nociceptors and that a translational approach to mouse afferent recordings in this study is feasible.

In addition to direct activation, the mechanical responses in mice were also tested before and after incubation of the receptive field with MMP-12 and it was found that in serosal layer afferents, there resulted in no change to mechanical sensitivity. In mesenteric receptive fields however, a significant increase in VFh probed response was observed. This response was abolished in the presence of a MMP-12-specific inhibitor.

The difference between responses in serosal and mesenteric afferents is not fully explored in this study although it may be a result of a differential expression of MMP-12 cleavage targets on afferent subtypes in different regions of the colon. For example, the ion channel $ASIC_2$ has shown preferential expression on mesenteric afferents and there is evidence for its activation from VFh probing (Page, 2005).

Although the MMP-12 responses on afferent firing were striking, the supernatant responses were much greater. In light of this, it was hypothesised that MMP-12 may have direct effects on afferent activation and may produce effects in combination with other inflammatory mediators. Therefore, an inflammatory soup (I.S) (BK, ATP, Histamine, 5-HT, and PGE_2) was added to serosal layer receptive fields twice over a 30 minute period. In control experiments, the second application of I.S resulted in a sharp reduction in afferent response compared with the first application. This is due to desensitisation of the afferent in response to high concentrations of inflammatory and pro-nociceptive mediators. Interestingly, when MMP-12 was pre-incubated over the receptive field prior to the second application of I.S this desensitisation was abolished. The second application produced a response greater than the first suggesting that the mechanism of action of MMP-12 involved a modulating activity on the afferent ending. Although this was not investigated further in this study, there is evidence that MMP's cleave voltage-gated sodium channels which may result in altered action potential kinetics (Remacle, 2015). In addition, MMP-12 has been shown to cause the release of ATP from epithelial cells which could have an additive effect on the afferent when I.S is applied (Gu & Lee, 2010). In line with the above observations, MMP-9, a protease also released from macrophages, was assessed in the same manner as MMP-12. It was observed that MMP-9, exogenously applied to receptive fields of mouse and human colonic afferents, would result in direct activation. When MMP-9 was pre-incubated over the receptive field before I.S application, it resulted in an increased level of firing, as was the case with MMP-12. Therefore, this study found that both MMP-9 and MMP-12 play a direct and indirect role in afferent activation and human nociceptor activation.

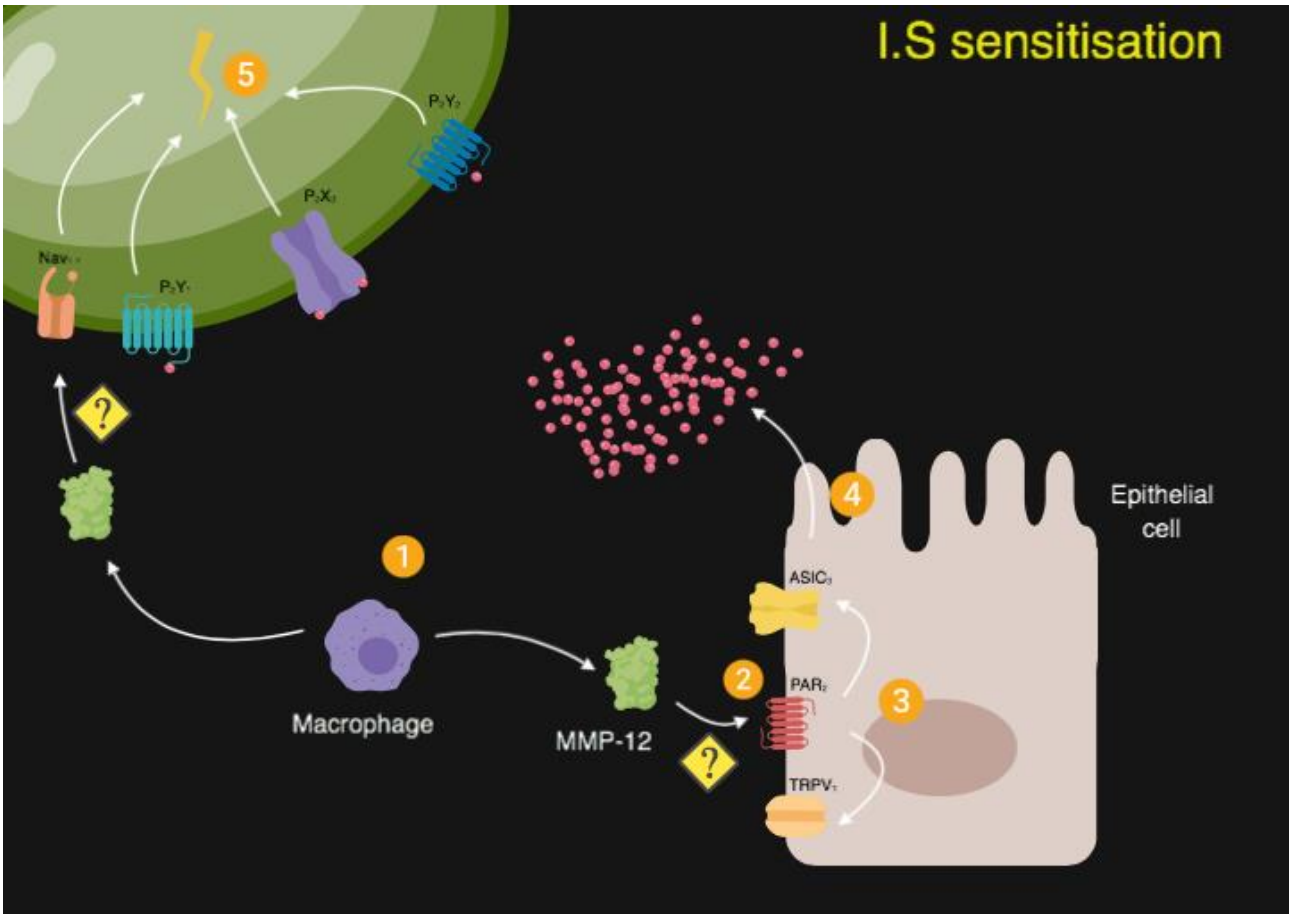


Figure 50. MMP-12 afferent sensitisation

This represents a proposed mechanism of MMP-12 effecting neuronal excitability in the presence of an inflammatory soup based only on current literature. MMP-12, released from macrophages, may act to induce ATP from epithelial cells through PAR activation to sensitise sensory afferents. The precise mechanism is unknown but throught to be linked with ion channel coupling. Additionally, binding sites for MMP's have been observed on the $Nav_{1.7}$ channel, which is known for it's role in threshold excitability in pain.

MMP's, including MMP-9 and MMP-12, were also significantly increased in UC biopsies and it is feasible that similar mechanisms of action may occur. However, correlating the expression level with afferent activity was not possible due to the presence of a small number of biopsy supernatants that appeared to inhibit afferent firing. Further analysis of transcript levels for potential inhibitory mediators found that high expression TIMP-1 and TIMP-2 expression was largely responsible for reduced afferent activity. This study suffers a limitation

whereby functional evidence for TIMP activity is not available and this should be taken into consideration. However, when coupled with the increased transcript expression of other protease inhibitors such as serpin A_3 and elafin, it remains highly credible that the influence of protease inhibition translates to a reduction in afferent firing. Therefore, this study suggests that proteases, specifically MMP-9 and MMP-12, play vital role in nociception in IBD and by increasing the activity of protease inhibitors, this may be reduced, thereby potentially targeting abdominal pain in IBD patients.

The main difficulties faced in this study are centered around the use of human biopsy tissue. The patient search, consent, and biopsy procedure, were all done by clinical staff. However, finding patients that met the criteria for this study (most notably pain levels for controls and patients) was challenging. Control patients undergoing biopsy procedure for polyp surveillance were difficult to obtain biopsies from due to the inclusion criteria requiring no recent abdominal pain. This therefore resulted in a low number of control patient biopsies leaving statistical analysis often open to type II errors.

Collection of FAPS, CD, and UC, biopsies was low throughput and proved a main difficulty early in the study. The biopsy incubation itself presented a main difficulty within this study. A low volume of supernatant was generated from each biopsy which meant that functional data on the presence and activity of MMP's was not attainable and transcript levels were often used as a surrogate marker for protein expression.

As mentioned above, the main limitation of this study is the reliance on transcriptional evidence for pro-nociceptive mediators. Although in many instances, evidence for a linear relationship between mRNA and protein exists, this study would undoubtedly benefit from protein measurements, in particular, the opioid mediators, and MMP's. The reliance on transcriptional data was a direct effect of the low volume of supernatant generated and as a result, functional evidence for the MMP and TIMP activity is lacking.

Future improvements would involve protein assessment from supernatants (duplicate biopsies collected if necessary) and full thickness human resected tissue. This would reduce the reliance on transcriptional evidence and support any observed changes in expression.

Future improvements that would result in additional supernatant or biopsies would also enable the application of fluorescence-activated cell sorting for immune cell analysis, which again would therefore not rely on transcriptional evidence as seen from this study.

In addition to future approaches mentioned above, a future direction of this study has set up a collaboration with an electrophysiology laboratory in Adelaide, Australia. The goal of this study is to identify whether TNBS mice present with increased expression of MMP-12 to enable the use of mouse tissue as a surrogate for human biopsy tissue. This would then enable higher volumes of supernatants. In addition, a future study would involve the addition of TIMP-1 and TIMP-2 to both human and rodent supernatants to understand a functional effect of TIMP inhibition.

Understanding the mechanism of action of both MMP-9 and MMP-12 on afferent activation would be a key future study. As we hypothesise that PAR_3 or $ASIC_2$ may influence MMP-driven effects, particularly mechanical changes, $PAR_3^{-/-}$ and $ASIC_2^{-/-}$ mice would enable this influence to be understood further.

The aim of this study was to identify novel mediators of abdominal pain in patients with FAPS, CD, and UC. It was observed that supernatants derived from patient biopsies were capable of eliciting an increased level of colonic afferent firing which we believe may represent the peripheral mechanisms involved in abdominal pain. Mechanical responses were also assessed after the application of supernatants but no changes were observed.

Biopsies from FAPS patients were mined for potential pro-nociceptive mediators which would result in abdominal pain in patients. Although various mediators have been linked to visceral pain in literature, this study did not find significant expression changes in FAPS biopsies compared with control biopsies. Therefore, an alternative approach to identifying pain mechanisms in FAPS meant the identification of $TRPV_4$ as an important ion channel in supernatant afferent responses. In $TRPV_4^{-/-}$ mice, FAPS supernatant responses were significantly effected and produced a marked reduction in afferent firing suggesting that $TRPV_4$ is important in the generation of action potential in colonic afferents from FAPS supernatants and could be considered a future target for research in abdominal pain.

After the observation that CD supernatants generate robust levels of afferent firing, the biopsy tissue from CD was assessed for both pro-inflammatory and pro-nociceptive mediators. Various mediators were increased but MMP-12 expression showed significant elevation and was found to correlate with afferent activity. MMP-9 analysis revealed a significant increase in expression compared with controls and was

examined to a greater extent. Further investigation showed that application of MMP-9 or MMP-12 to both rodent colonic afferents and human nociceptors caused afferent firing suggesting a direct mechanism of nociception from this protease. MMP-12 application also enhanced the mechanical sensitivity to mesenteric afferents although did not appear to alter serosal layer sensitivity. Application of either MMP-9 or MMP-12 sensitised colonic afferents to an inflammatory soup suggesting it has a modulatory mechanism on afferent endings in combination with direct activation.

UC biopsy investigation revealed identical expression patterns of MMP's but identified a subgroup of patient biopsies that were considered to have above normal expression of inhibitory mediators. Further investigation found that the protease inhibitors serpin A_3, elafin, TIMP-1 and TMIP-2, were greatly increased in this subgroup of biopsies and that they likely contributed to a reduction in afferent firing. Therefore, this study suggests that enhanced regulation of protease activity, particularly MMP-9 and MMP-12, through the use of TIMP-1 and TIMP-2 especially, is likely to offer a protective role in abdominal pain in IBD patients.

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APPENDIX A: PROTEASE ACTIVATED RECEPTOR

PAR_4

Protease receptors, PAR_{1-4}, are widely expressed on mast cells, B-cells, afferent endings, colonic epithelium and DRG neurons while PAR_2 activation has largely been shown to mediate mechanical hypersensitivity through coupling mechanisms with the cation channel $TRPV_4$, PAR_4 has been proposed to have the opposite effect by inhibiting the PAR_2 and $TRPV_1$ activation, and been classed largely as inhibitory (Moormann, 2006; Vergnolle, 2010; Augé, 2009; Asfaha, 2007).

A study from the Cenac laboratory observed the visceromotor response (VMR) in mice when given PAR_2 or $TRPV_1$ agonists, and observed the VMR when pretreated with a PAR_4 ligand to show a decrease not only in the response but that it prevented allodynia and hyperalgesia from the PAR_2 and $TRPV_1$ agonists (Augé, 2009; Amadesi, 2006). PAR_4 activation can also lead to inhibitory effects when PAR_2/$TRPV_4$ pathways are activated, resulting in reduced VMR responses (Augé, 2009).

In addition to this, PAR_4 has been shown to be anti-nociceptive in TNBS mice and patch clamp recordings have also shown that PAR_4 activation in rodent DRG colonic neurons can suppress the excitability, and local analgesic effects have been reported, to thermal and mechanical stimuli when injecting PAR_4 ligands into the paws of rodents (Annaházi, 2012; Asfaha, 2007).

Table 6. The direct effect of PAR4-AP on afferent firing.

Recording	Concentration (μM)	VFH Change (%)	Mean change in firing (%)	N	Mean change in firing -post 10 min (%)
Single unit	10μM	-13.9%	-32.3%	7	N/A
Whole nerve	30μM	N/A	-9.4%	5	-9.4%

In this study, PAR_4 agonist PAR_4-AP was added to mouse serosal afferents, or a whole splanchnic nerve. Both mean firing and mechanical responses were observed. However, further investigations, in particular with the use of PAR_4-AP in combination with biopsy supernatants would be beneficial.

APPENDIX B. CALCIUM IMAGING FOR MMP-12

DRG removal and culture

DRG removal and culture was done by researcher Egle Gaurilcikaite at Kings College London, UK. Mice were killed by overdose of sodium pentobarbital and the spinal cord removed. Thoracic dorsal root ganglia (DRG) were removed and incubated in F12 medium (Gibco, Paisley, UK) with 1.25% collagenase for 60 minutes at 37℃. Neurons were pelleted by centrifugation for 6 minutes at 60 x g before being resuspended in F12 medium (1% penicillin/streptomycin, 30% BSA, 1% N2 neuronal growth supplement; PAA, Yeovil, UK). DRG neurons were then plated onto a sterile coverslip coated with poly-L-lysine (100µg/mL^{-1}; Sigma, Dorset, UK) and laminin (10µg/mL^{-1}; Sigma, Dorset, UK) and cultured overnight (18-24 hours) for calcium imaging experiments.

Calcium imaging

Calcium imaging was done by myself under the supervision of researcher Egle Gaurilcikaite at the Kings College London laboratory. Cells were incubated in buffer (HBSS with 10mM glucose, 10mM HEPES, pH 7.4) with Fura-2-AM (2mM) and probenecid (1mM) for 60 minutes at 37°C. Coverslips were washed and mounted in an open chamber. MMP-12 protein (20pM, 50pM, 100pM) was diluted to required concentrations with buffer and applied to cells by continuous perfusion for 30 seconds at 37°C. The fluorescence of cells was measured at 340 and 380nm excitation and 510nm emission using microscope imaging (PTI, Ford, UK). Vehicle for MMP-12 activation was tested at 100nM and 1µM concentrations. Cells were challenged with KCl (50mM) at the end of each experiment to activate voltage gated Ca^{2+} channels and provide a maximal Ca^{2+} signal, against which to normalise other cellular responses.

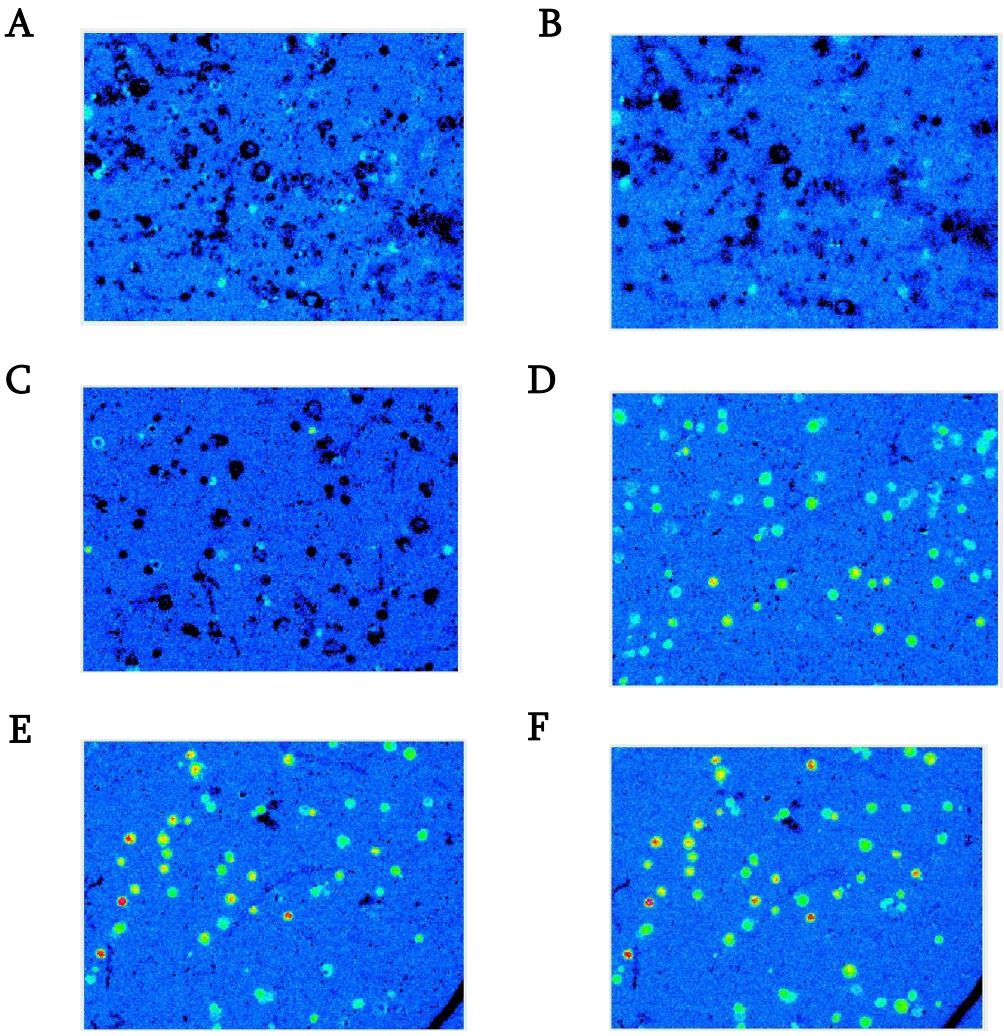

Figure 51. Calcium imaging dose response for MMP-12.

(A) The baseline activity before the addition of test products. **(B)** Application of 1µM vehicle (APMA) with no DRG activation. **(C)** Application of MMP-12 protein (20pM) with 8.95% of DRG's showing activation. **(D)** Application of MMP-12 protein (50pM) with activation in 74.38% of DRG's. **(E)** Application of MMP-12 protein (100pM) with 82.88% of DRG's activated. **(F)** Application of KCl following MMP-12 dose studies.

APPENDIX C. APMA VEHICLE CONCENTRATIONS

Following calcium imaging studies which were able to demonstrate intracellular calcium increases in response to MMP-12, the full length protein MMP-12 was added to colonic afferents. Before the MMP-12 could be activated with APMA and used experimentally, a concentration-response curve for APMA was

tested. The APMA was diluted to 100nM, 1µM, 10µM, and 100µM, with Krebs buffer and pre-warmed to 37℃. Next, following a 3 minute baseline recording period, the APMA was placed inside a metal ring placed over the afferent receptive field for 7 minutes. Finally, the APMA and ring were removed and wavemark analysis using Spike2 software compared afferent firing. The APMA resulted in no increase in afferent firing compared with previous vehicle control responses (Krebs/ Hepes).

Table 7. APMA concentrations with afferent firing responses

APMA Concentration	Change (Spikes/s^{-1})	N
100nM	0.07 ± 0.02	3
1uM	-0.05 ± 0.06	3
10uM	0.06 ± 0.10	3
100uM	0.06 ± 0.03	6

APPENDIX D. QUANTITATIVE PCR PRIMERS

The taqman primers were chosen from an inventoried catalogue (thermofisher scientific, uk) where the primer sequence is not disclosed. However, the primer identification numbers for all genes of interest are presented in the table below.

Table 8. The primer identification for qPCR

Gene	Inventoried Assay ID		Gene	Inventoried Assay ID
MMP-1	Hs00233958_m1		IL-17A	Hs00174383_m1
MMP-3	Hs00968305_m1		FOXP3	Hs00203958_m1
MMP-9	Hs00234579_m1		CD14	Hs00169122_g1
MMP-12	Hs00899662_m1		CD16	Hs04188274_m1
MMP-19	Hs00418247_g1		TLR4	Hs00152939_m1
TIMP-1	Hs00171558_m1		NEUTROPHIL ELASTASE	Hs00975994_g1
TIMP-2	Hs00234278_m1		COX-1	Hs00377726_m1
TIMP-3	Hs00165949_m1		COX-2	Hs00153133_m1
TIMP-4	Hs00162784_m1		PRO-ENKEPHALIN	Hs00175049_m1
TRYPTASE B2	Hs02576518_gH		POMC	Hs00174947_m1
HISTIDINE DECARBOXYLASE	Hs00157914_m1		PRO-DYNORPHIN	Hs00225770_m1
TRYPTOPHAN HYDROXYLASE I	Hs00188220_m1		ELAFIN	Hs00160066_m1
CATHEPSIN G	Hs01113415_g1		SERPIN A3	Hs00153674_m1
IL-1β	Hs00174097_m1		SERPIN B1	Hs00961948_m1
TNFα	Hs00174128_m1		SOMATOSTATIN	Hs00356144_m1
IL-6	Hs00174131_m1		GAPDH	Hs99999905_m1
IL-10	Hs00174086_m1		β-ACTIN	Hs99999903_m1
IFNγ	Hs00174143_m1		18s	Hs99999901_s1
IL-8	Hs00174103_m1			

Table 9. Electrophysiology raw data

Patient number	Patient Phenotype	1.0g VFH Spikes/s-1 PRE -	1.0g VFH Spikes/s-1 POST -	Baseline Mean Spikes/60s	Incubation Mean Spikes/60s	Change Mean Firing spikes /60s	Change Firing spikes /second	Baseline Rate/ spikes/ 60/s MAX	Incubation Rate/spikes/ 60s/ MAX	Change PEAK firing spikes//60s
1.1	CD	N/A	N/A	28.7	89.6	60.9	1.02	33	97	64
9.1	CD	12.11	N/A	25	27.71	2.71	0.05	34	34	0
9.2	CD	27.22	38.11	1.3	1	-0.3	-0.01	2	2	0
21.1	CD	5.89	7.11	0.33	25	24.67	0.41	1	47	46
21.2	CD	12.22	15.33	9.33	30.57	21.24	0.35	11	36	25
24.1	CD	5.89	5.78	0.33	36.43	36.1	0.60	1	47	46
24.2	CD	5.44	4.44	19.33	45.00	25.67	0.43	23	54	31
28.1	CD	7.44	7.44	30.33	77.29	46.96	0.78	36	92	56
28.2	CD	9.11	7.0	1	12.43	11.43	0.19	2	17	15
29	CD	6.89	9.67	2	26.14	24.14	0.40	4	38	34
32.1	CD	13.11	8.22	20.33	0.29	-20.04	-0.33	26	2	-24
32.2	CD	4.56	4.44	0	11.57	11.57	0.19	0	24	24
19-1	CD	7.78	8.78	0.33	56.43	56.1	0.94	1	54	53
19-2	CD	5.67	8.33	2.67	0.71	-1.96	-0.03	5	3	-2
37-1	CD	6.56	9.78	6.67	38.86	32.19	0.54	10	48	38
37-2	CD	N/A	N/A	16.67	10	-6.67	-0.11	11	36	25
38-1	CD	8.44	10.22	101.67	91.86	-9.81	-0.16	112	110	-2
38-2	CD	N/A	N/A	40.33	71.57	31.24	0.52	63	82	19
1.2	CD	18.56	18.44	0.00	0.00	0.00	0.00	0	0	0
2	CD	8.33	11.78	18.67	56.57	37.9	0.63	27	76	49
4	CD	7.89	9.78	2	57.29	55.29	0.92	7	63	56
12	CD	14	10.44	53.33	35.29	-18.04	-0.30	74	51	-23
27.1	CONTROL	4.78	5.22	0.50	7.57	7.07	0.12	2	20	18
27.2	CONTROL	4	3.89	0	14.14	14.14	0.24	0	29	29

20-1	CONTROL	19.33	N/A	1	5.86	4.86	0.08	1	9	8
20-2	CONTROL	10.44	11.0	11.67	26.57	14.9	0.25	15	33	18
27.3	CONTROL	N/A	N/A	0	13	13	0.22	8	32	24
33-1	CONTROL	13.56	13.11	0	4	4	0.07	0	8	8
33-2	CONTROL	4.67	4.33	0.25	10.71	10.46	0.17	1	21	20
33.3	CONTROL	N/A	N/A	13	30	17	0.28	17	33	16
36-1	CONTROL	N/A	N/A	2	8.42	6.42	0.11	26	105	79
36-2	CONTROL	5.56	4.67	1.33	26.86	25.53	0.43	10	51	41
6.2	FAPS	9.56	N/A	17.0	89.0	72.0	1.20	26	117	91
7.1	FAPS	9.22	13.22	1.3	41.14	39.84	0.66	4	57	53
7.2	FAPS	10.1	N/A	36.67	95.43	58.76	0.98	43	126	83
8.2	FAPS	N/A	N/A	20.33	11.71	-8.62	-0.14	25	22	-3
10.1	FAPS	N/A	N/A	6.3	7.14	0.84	0.01	21	17	-4
10.2	FAPS	9.67	N/A	13.33	43	29.67	0.49	23	73	50
14.1	FAPS	6.67	5.78	2.33	2.14	-0.19	-0.00	5	4	-1
14.2	FAPS	6.89	7.67	10.0	21.57	11.57	0.19	13	36	23
16	FAPS	9.56	8.67	9.67	20.86	11.19	0.19	17	33	16
22.1	FAPS	13.56	6.44	2.67	41.29	38.62	0.64	3	58	55
22.2	FAPS	8.33	10.22	28	47.14	19.14	0.32	28	63	35
23	FAPS	8.11	6.78	16.67	20.29	3.62	0.06	19	32	13
26	FAPS	N/A	N/A	3.00	23.71	20.71	0.35	4	35	31
18-1	FAPS	7.78	7.22	0	4.57	4.57	0.08	0	9	9
18-2	FAPS	7.78	17.4	12.67	107.1	94.43	1.57	18	119	101
6-3	FAPS	7.56	7.11	45.3	45.1	-0.19	-0.00	47	56	9
3	FAPS	8.78	5.33	5.67	31.14	25.47	0.42	11	50	39
6.1	FAPS	18.67	15.44	0	0	0	0.00	0	0	0
5.2	UC	8.22	10.33	0	0	0	0.00	0	0	0
11.1	UC	10.11	14.78	6.67	24.29	17.62	0.29	25	40	15

11.2	UC	5.60	5.78	4.33	4.0	-0.33	-0.01	9	13	4
13.2	UC	6.11	6.33	7	36.86	29.86	0.50	11	12	1
15.1	UC	8.56	7.33	15.0	32.0	17.0	0.28	21	48	27
15.2	UC	7.22	6.56	16.33	84.71	68.38	1.14	24	127	103
17.1	UC	N/A	N/A	24	23	-1	-0.02	9	31	22
17.2	UC	9.0	6.33	0.33	2.42	2.09	0.03	1	7	6
25.2	UC	6.56	6.56	19.67	38.29	18.62	0.31	24	45	21
30	UC	9.11	10.33	30	35.3	5.3	0.09	17	23	6
31	UC	N/A	N/A	22.67	11.29	-11.38	-0.19	7	13	6
34-1	UC	N/A	N/A	11.2	17.4	6.2	0.10	74	57	-17
34-2	UC	7.78	7.67	21.67	53.43	31.76	0.53	27	80	53
35-1	UC	8.67	12.67	49	90.86	41.86	0.70	60	165	105
35-2	UC	N/A	N/A	14.33	41.14	26.81	0.45	0	8	8
5.1	UC.	8.00	6.44	12.33	36.00	23.67	0.39	19	49	30
13.1	UC.	N/A	N/A	3	0	-3	-0.05	1	18	17